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Development of a DNA Probe for the Rapid Detection of Cytomegalovirus

Nell Lurain
Loyola University Chicago

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DEVELOPMENT OF A DNA PROBE
FOR THE RAPID DETECTION
OF CYTOMEGALOVIRUS

by
Nell Lurain

Library -- Loyola University Medical Center

A Thesis Submitted to the Faculty of the Graduate School of
Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

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LIFE

The author, Nell S. Lurain, is the daughter of William P. Snaveley and Alice (Pritchett) Snaveley. She was born August 1, 1946, in Charlottesville, Virginia.

Her secondary education was obtained at the Edwin O. Smith High School, Storrs, Connecticut, where she graduated in June, 1964. She attended Oberlin College, Oberlin, Ohio and received the degree of Bachelor of Arts with a major in biology in June, 1968. She then attended the University of Virginia where she received a Certificate in Medical Technology in September, 1969.

From 1969 to 1972 she worked as a medical technologist in the Clinical Microbiology Laboratory at North Carolina Memorial Hospital, Chapel Hill, North Carolina and from 1972 to 1975 in the Clinical Microbiology Laboratory at Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania.

From September, 1977 to June, 1979, she was enrolled as a part-time graduate student in the Department of Medical Technology at the State University of New York, Buffalo, New York. In January, 1980, she entered the graduate program of the Department of Microbiology of Loyola University at the Medical Center.

Ms. Lurain is the co-author of the abstract: N. Lurain and K. Thompson, "Development of a probe for rapid detection of cytomegalovirus by DNA-DNA hybridization", Annual Meeting of the American Society of Microbiology, 1984.

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LIST OF ABBREVIATIONS

AIDS.....	Acquired immunodeficiency syndrome
ATP.....	Adenosine triphosphate
BSA.....	Bovine serum albumin
cm.....	Centimeter
CMV.....	Cytomegalovirus
CPE.....	Cytopathic effect
CTP.....	Cytidine triphosphate
cpm.....	Counts per minute
DAB.....	Diaminobenzidine tetrahydrochloride
DMSO.....	Dimethyl sulfoxide
DNA.....	Deoxyribonucleic acid
DTT.....	Dithiothreitol
EBSS.....	Earle's balanced salt solution
EDTA.....	Ethylenediaminetetraacetic acid
FCS.....	Fetal calf serum
g.....	Gram
h.....	Hour
hrp.....	Horse-radish peroxidase
HSV.....	Herpes simplex virus
IR _L or S.....	Inverted repeat (long or short)
kb.....	Kilobases or kilobase pairs
L.....	Liter
M.....	Molar

μCi.....	Microcurie
μg.....	Microgram
mg.....	Milligram
min.....	Minute
μl.....	Microliter
ml.....	Milliliter
mM.....	Millimolar
MOI.....	Multiplicity/multiplicities of infection
ng.....	Nanogram
PEG.....	Polyethylene glycol
pfu.....	Plaque-forming units
pg.....	Picogram
PVP.....	Polyvinylpyrrolidone
RNA.....	Ribonucleic acid
RNase.....	Ribonuclease
SDS.....	Sodium dodecyl sulfate
SSC.....	Standard saline citrate
U _L or S.....	Unique (long or short)
v/v.....	Volume/volume
VZV.....	Varicella-zoster virus
w/v.....	Weight/volume

INTRODUCTION

Structure and Lytic Cycle. Cytomegalovirus (CMV) is an enveloped double-stranded DNA virus belonging to the family Herpesviridae. Other human viruses which are classified in this family include Epstein-Barr virus (EBV), herpes simplex types 1 and 2 (HSV-1, HSV-2) and varicella-zoster (VZV). The growth characteristics of these viruses in tissue culture differ with respect to permissive cell type, length of replication cycle and extracellular viral titer (20). HSV types 1 and 2 grow in a variety of mammalian cells with a short replication cycle resulting in release of the virions into the extracellular fluid. By comparison CMV is highly species-specific. Wild-type human CMV (HCMV) replicates very slowly only in human fibroblasts with little or no release of virus into the tissue culture fluid. Repeated passage of HCMV isolates in culture may eventually result in measurable viral titer in the extracellular fluid. The titer, however, varies with the particular strain.

The complete virion of CMV is composed of a double-stranded linear DNA-containing core, an icosahedral capsid and an envelope (123). Electron micrographs, however, have demonstrated the presence of many incomplete viral particles. In general four morphological types are present: naked empty cores, naked "full" cores, enveloped empty cores and enveloped "full" cores corresponding to intact virions (37,44,144). Another characteristic form found in CMV-infected cells is the "dense body", which appears to be excess viral structural proteins with an envelope. The dense bodies carry CMV-specific antigens but have no detec-

table DNA (56,110,124).

Electron microscopy has also been used to compare the replication steps of HSV and CMV. Both viruses enter cells by phagocytosis or fusion with the plasma membrane. Both rapidly traverse the cytoplasm to the perinuclear area, but CMV nucleocapsids appear to acquire a fine fibrillar coat in the cytoplasm and are subsequently disassembled much more slowly than HSV nucleocapsids (4, 113). Adsorption and penetration, therefore occur equally rapidly in both HSV and CMV infections, but subsequent steps in viral replication are much slower for CMV than for HSV (20, 137).

Epidemiology. The slow rate of CMV replication and the highly cell-associated nature of the virus have presented problems for diagnosing human infections. CMV is an ubiquitous virus as demonstrated by the high percentage of seropositive persons in various social groups. Detectable antibody titers have been found in 53% of children in a day care center (90), 12% of a group of pregnant women (103), 80% of the general Swedish population (139) and 94% of homosexual men (28). Most infections are asymptomatic, but serious complications may occur following prenatal, perinatal or iatrogenically-induced exposure. This last group of patients includes organ transplant and transfusion recipients (19,46,70,87,96,104).

Recently CMV has been linked with the acquired immune-deficiency syndrome (AIDS) observed mainly among drug users, male homosexuals, Haitians and hemophiliacs (24,28,42,82,89,132). Many of these patients have detectable CMV antibody titers, and often the virus can be isolated from clinical specimens (48,42) in some cases in the absence of a rising antibody titer to CMV (29). Other opportunistic infections such as

Pneumocystis carinii and Candida albicans are also frequently present, thus the exact role of CMV in this new disease has not been established. In a recent study disseminated CMV infection was found at autopsy in 14 out of 15 AIDS patients. This suggests that CMV viremia may be in many cases the immediate cause of death (79), although CMV is probably not the agent which produces the initial immunodeficiency (38).

Congenital infection depending on the severity may result in a range of symptoms from death to persistent viruria. The central nervous system (CNS) is most often affected. Confirmation of congenital infection requires isolation of virus from specimens such as urine taken during the first week of life (27,119). The source of the virus appears to be predominately a reactivation of maternal infection, although occasionally primary maternal disease occurs during pregnancy (121,139). The severity of symptoms is greatest with primary maternal infection, but factors such as gestational age at the time of infection and the immune response of the fetus may also contribute to the clinical outcome (10,91,92,120,121).

Perinatal infection is also usually maternal in origin, but nosocomial sources may produce disease in some cases (51,117,139). The maternal infection is again generally a reactivation of latent virus (61) and is passed to the infant through the genital tract or in breast milk (62,90,103). In contrast to congenital infection, the CNS is not the most frequently affected organ. Instead perinatal disease may be detected in the liver, spleen, kidneys and hematopoietic tissues. In some cases the children have neurologic sequelae even though they are initially asymptomatic. Slow psychomotor development and loss of hearing

may become apparent at a later age (139). Again as with congenital disease the severity of perinatal CMV infection is greater in association with primary maternal infection (121).

In adults CMV is often isolated from multiple transfusion recipients and renal, cardiac, or bone marrow transplant patients. Viruria, cytomegalic inclusion pneumonia and heterophile-negative mononucleosis are the most common CMV-related diseases found in these patients (25,70,95,139). Whether the disease represents reactivation of latent virus or exogenous donor infection has not been completely determined. CMV has been recovered from the buffy coat of donor blood (25,61); multiply-transfused patients show a higher rate of seroconversion, and the timing of the rise in antibody titer is not characteristic of an anamnestic response (96). It would appear, therefore, that donor blood can be a source for CMV transmission. The possibility of reactivation of latent infection cannot be ruled out, however, since allogeneic stimulation of infected lymphocytes has been observed in mice (70,87). In addition, molecular analysis of CMV strains from a group of blood donors and their recipients has demonstrated a lack of relatedness between the corresponding viral isolates (61).

Organ transplant recipients appear to be very susceptible to CMV infection. In the majority of cases the source of the virus is probably a reactivation of latent host infection. In one study of renal transplant recipients all patients who developed active CMV infections had detectable CMV antibody before receiving renal allografts (106). The CMV viremia was associated with graft dysfunction.

In a series of cardiac transplant patients (95) the donors were

demonstrated to be the source of CMV when the recipients were seronegative for the virus. These recipients had primary CMV infection from a reactivation of latent virus in donor tissue or contaminating blood. The recipients who were seropositive for CMV before transplantation and who subsequently developed CMV viremia, most likely had a reactivation of latent host virus, although reinfection from a nosocomial source has been reported (133).

CMV infection in transplant or transfusion recipients, therefore, often occurs in spite of measurable serum antibodies (30,46,54,95,96,104), and the outcome may depend on the cellular immune response, which is usually depressed in the presence of CMV (12,32,99,100,108,136). Inversion of the T-cell helper/inducer ratio in patients following transplantation may be predictive of the risk of developing CMV infection (111). Active CMV infection may cause immunosuppression (32,102,108). Therefore, efforts to determine the exact pathogenesis of CMV in transplant recipients have not produced conclusive answers.

Latency and Oncogenicity. Since latency of CMV appears to play a significant role in the pathogenesis of the virus for these immunocompromised patients, there have been a number of studies to determine the site of the latent infection. Polymorphonuclear and adherent mononuclear leukocytes seem to be the most likely sources of inactive virus (25,30,31,67,68,107), although cultivation of cells from other sources has occasionally resulted in productive viral infection (143) or detectable CMV antigens (101). Factors which may trigger activation of the virus include another acute infection (69), host immunosuppression (70) or host response to allogeneic cells (87).

In addition to its ability to produce latent infection, CMV may abortively infect nonpermissive cells (9,37,39,101). Abortive infection may result in stimulation of cellular DNA and RNA synthesis (2,22,37). Early viral antigens are expressed, but viral DNA cannot be detected (23,75). Similar abortive infections are characteristic of oncogenic DNA viruses such as SV-40, polyoma and adenovirus (37).

There is evidence that CMV also is potentially oncogenic (1,2). Albrecht and Rapp (1) infected nonpermissive hamster embryo fibroblasts with a human CMV strain and obtained a cell line which produced tumors in weanling hamsters. After animal passage, however, no virus could be rescued, although the tumor-bearing animals had antibodies to CMV antigens. Another group of investigators (101) grew a cell line from in vivo CMV-infected human prostate cells to passage levels well above normal. Again no virus could be rescued, the karyotype was diploid and no tumors were produced in nude mice. The cells were, however, no longer contact-inhibited, and CMV-specific antigens were expressed. Geder et al. (39) reported transformation of human embryonic lung cells infected at a low multiplicity of infection (MOI) with CMV. Their cell line induced tumors in nude mice, expressed CMV-specific membrane antigens and underwent uncontrolled nuclear divisions in the presence of cytochalasin B.

The outcome of CMV infection, therefore, may be influenced by the type and species of cell, the physiological state of the cell (19,22) and the temporal expression of the viral genome (20,125,137,138). Three groups of virus-specific proteins have been described based on their order of appearance and expression in the infected cell. Immediate early

polypeptides (IEP) are produced in lytically-infected cells after treatment with protein synthesis inhibitors or in abortively-infected cells (45,64,127). There is a switch from IEP to early proteins (EP) within two hours postinfection in lytically-infected cells. Early proteins are also expressed in the presence of viral DNA inhibitors and in latently-infected cells (6,44). Late proteins (LP) require viral DNA synthesis for expression and are, therefore, not produced in nonpermissive, abortively or latently-infected cells (6,43,83). Such temporal control of genome expression is typical of DNA viruses. The regulatory proteins involved may be responsible for the prolongation of the CMV lytic cycle and for the determination of latency or persistence in non-lytic infections (137).

Molecular Characteristics of CMV DNA. Molecular analysis of the DNA of different HCMV isolates has been used to study the different types of viral infection. It is now known that the size of the infectious HCMV genome is approximately 150×10^6 daltons with some variation among individual strains (19,40,126). Early studies had reported that the CMV genome was 100×10^6 daltons, which is the size of HSV DNA. These reports also mentioned a less abundant class of molecules of 150×10^6 daltons (19,57,72,76). Stinski et al. (126) demonstrated that a low MOI resulted in production of the majority of viral DNA molecules of the larger size class. Thus, the 100×10^6 dalton species represented defective genomes, which appeared in larger numbers when the MOI was greater than 1.

The structure of the CMV genome is similar to that of the herpes simplex viruses in spite of the difference in size. There is a long

unique sequence (U_L) bounded by inverted repeats (IR_L) and a shorter unique sequence (U_S) also bounded by a different set of inverted repeats (IR_S) (Fig. 1). The presence of submolar fragments in restriction endonuclease digests of the viral DNA supports a model in which there are four possible genome arrangements. Inversion of each unique sequence and its repeats relative to the other produces the four structures, which are present in approximately equal amounts in infected cells (15,49,72, 141).

Although HSV 1 and 2 and CMV have structurally similar genomes they appear to lack homology. Renaturation kinetics by DNA-DNA hybridization has demonstrated less than 5% homology between HCMV strains and HSV. Cytomegalovirus also does not share homology with other herpes viruses such as EBV (58).

Restriction endonuclease cleavage patterns and nucleic acid hybridization techniques have been used to determine strain variation of CMV (61,115,128). Strains of CMV from the same species show approximately 80% or greater homology of the DNA genome (59,97), and restriction endonuclease site polymorphism has been used to determine the epidemiology of exogenous and endogenous infections (26,62,84). Comparisons of CMV strains using several restriction endonucleases have provided strain-specific "fingerprints". Huang et al. (62) studied a group of women and their infants who had evidence of CMV infection within the first year of life. The pattern of DNA restriction fragments of mother and offspring were either identical or showed very little variation. Repeat isolates from some of the same patients over a period of as long as nine years showed stability of the viral genome responsible for the

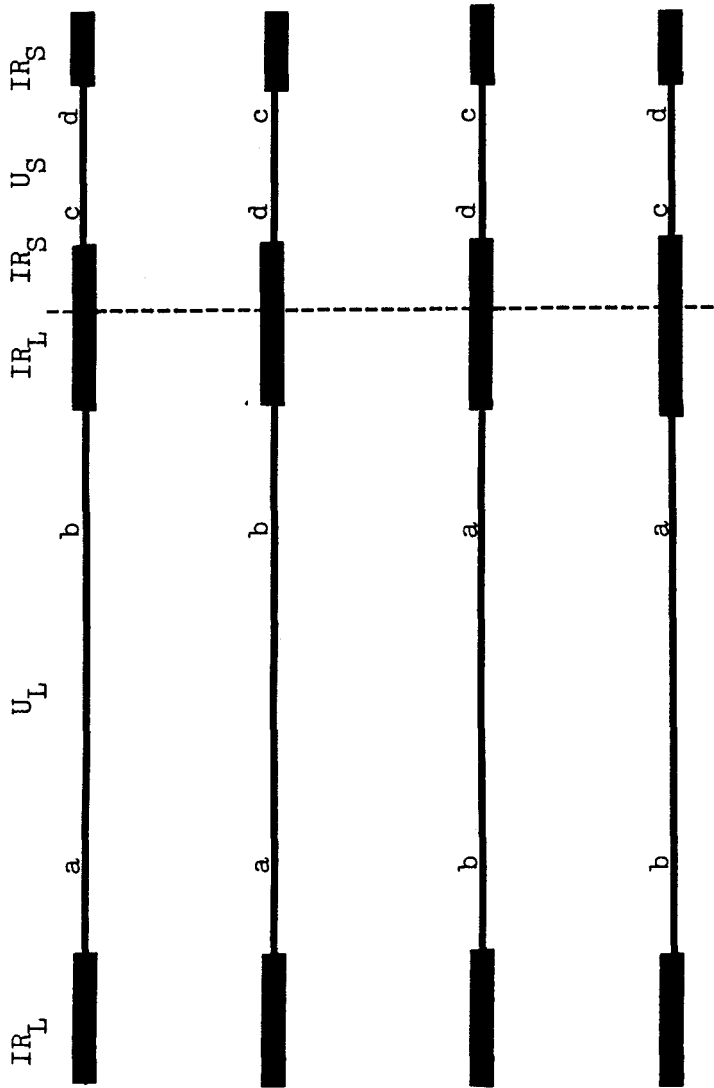
Figure 1. The structure of the genome of HCMV. The four arrangements are generated by the ability of each unique sequence and its associated repeats to invert in relationship to each other.

IR_L = long inverted repeat sequence

IR_S = short inverted repeat sequence

U_L = long unique sequence

U_S = short unique sequence



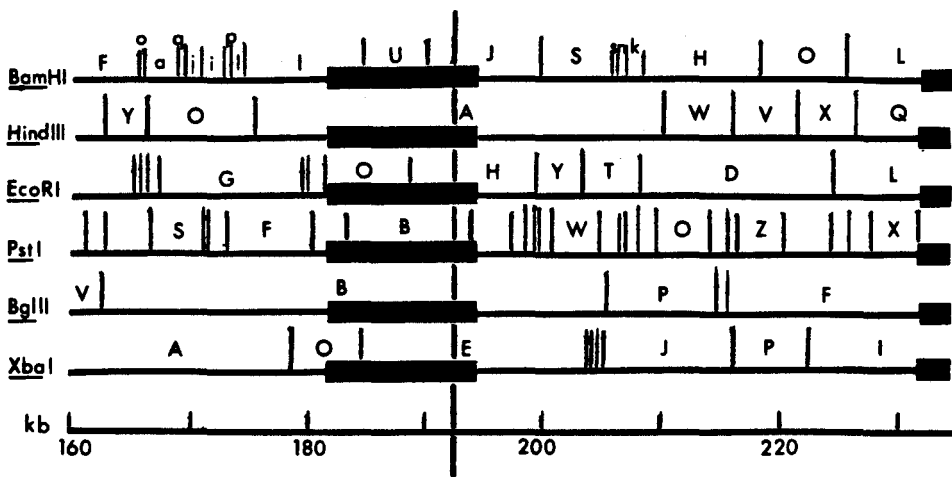
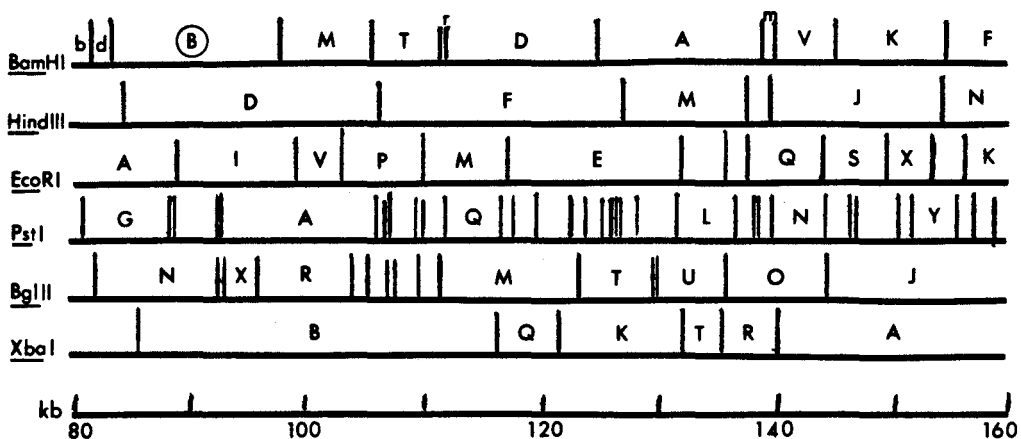
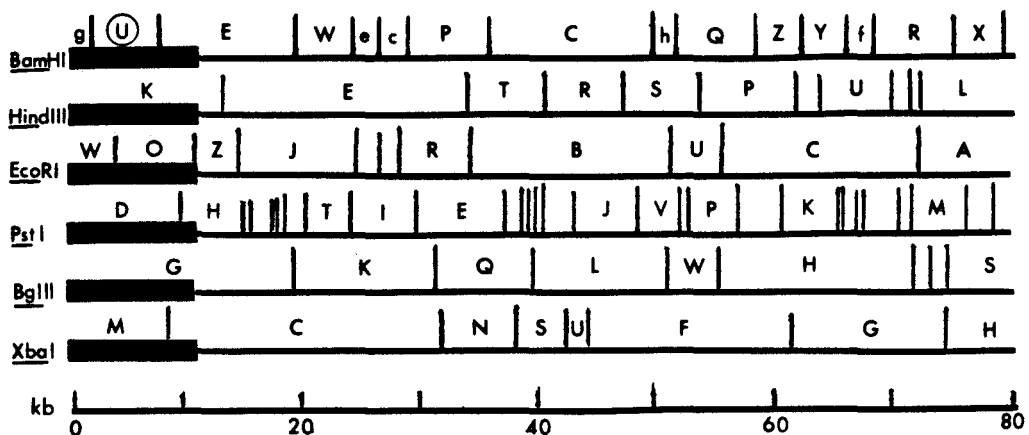
initial infection. Only CMV isolates from the same or related persons were essentially identical. Unrelated controls carried heterogeneous strains of CMV. In spite of the apparent strain stability, however, there is as yet no classification of CMV similar to that of HSV, which can be separated into two types by both antigenic and molecular criteria (71,123).

Several groups of investigators have mapped and cloned the genome of the common laboratory strains of HCMV such as Davis (21), Towne (74) and AD169 (49,130). Specific cloned fragments of the DNA have been used to look for homologous sequences in cellular DNA and other herpesviruses. From these studies it has been determined that there are regions of homology with cellular DNA, and that certain cloned fragments are able to transform cells.

Nelson et al. (85) identified a 2.9 kilobase (kb) region of the AD169 genome, which was capable of transforming NIH 3T3 cells. This region is about 20 kb from the left end of the U_L sequence (Fig. 2). The cells transfected with plasmids containing this part of the genome displayed anchorage independence and tumorigenicity in nude mice. The transcription of this region corresponds to an area of IEP messenger RNA, although no translation product has been isolated. Sequencing data indicate that there is no viral protein product from this region, therefore, the mechanism of transformation is not known (86).

A second transforming region of the CMV genome has been reported by Clanton et al. (14). This region shows homology to one of the transforming regions of HSV-2, when hybridization is performed under non-stringent conditions. The cloned fragment from the Towne strain of CMV

Figure 2. Restriction enzyme map of CMV AD169 genome.
From Greenaway et al. (48)



hybridized to a unique fragment of AD169, but this was also different from the transforming fragment described by Nelson (85). The Towne fragment was able to transform Syrian hamster embryo cells. Immortal lines were produced which grew in 2% serum and displayed anchorage independence. There appear, therefore, to be two separate transforming regions in the HCMV genome both of which show some homology to transforming regions previously found in HSV-2 (14).

Spector and Vacquier (116) described nucleotide sequences related to the transforming gene (v-myc) of avian myelocytomatosis virus, which were present in the genome of AD169. There were several regions in the U_L segment and the IR_S , which showed homology to the 5' end of the v-myc. These regions, however, did not correspond to the transforming region identified by Nelson et al. (85). Since the v-myc sequence is homologous to cellular oncogenes (c-myc), these authors postulate that there may be early activation of c-myc followed by activation of another set of genes to produce cell transformation.

To investigate further the relationship of these transforming regions with the known cellular and viral oncogenes (c-myc and v-myc), Gelman et al. (41) showed that myc-homologous sequences were present in subfragments of one of the Towne transforming fragments (14). These regions of homology in the HCMV genome, however, corresponded only to the 5' half of the c-myc and v-myc sequences. Whether or not the virus acquired these sequences by recombination with human DNA cannot yet be determined.

There are sequences found in the intermediate repetitive class of mammalian cellular DNA, which hybridize weakly to CMV Towne DNA frag-

ments containing the junction between the long and short segments and to fragments carrying the termini of the long inverted repeat (93). Similar cell-virus DNA homology has been demonstrated for HSV 1 and 2 (93,98). The inverted repeats of HSV 1 and 2 also show the strongest hybridization with cellular DNA (93).

Taken together these studies of the CMV genome indicate that some homology exists between certain segments of the viral DNA and mammalian cellular DNA. Some of these sequences may be responsible for cell transformation, but what role they might play in lytic or latent CMV infection is still unknown.

Rapid Detection of CMV. The potentially severe consequences of CMV infection make rapid diagnosis desirable. Routine virus cultures of patient specimens for CMV often require two weeks or longer to produce visible cytopathic effect (CPE) (27). Serodiagnosis is the only currently available technique for detecting CMV infection in the absence of a positive virus culture (35,36,50,56,131). The two techniques which show the most promise for rapid viral identification are: 1) detection of early viral antigens with monoclonal antibodies, and 2) hybridization of viral nucleic acids with labelled probes.

Monoclonal antibodies specific for CMV-infected cells are available (47,73,94,135). Strain AD169 was used to produce the antibodies, but cross-reactivity with other strains of CMV from both laboratory and clinical sources demonstrated the potential for universal detection of CMV infections. Several of the antibodies reacted with early CMV glycoproteins (47,73), which could allow rapid detection of the virus in cell culture. An antibody reacting with a late glycoprotein (47) also

was able to detect virus directly in clinical specimens. These reports indicate that it may be possible to develop monoclonal antibodies for antigens present early in infection by all strains of HCMV.

For this project the technique of nucleic acid hybridization has been chosen as a possible method for rapid detection of CMV. Since there is such a large percentage of DNA homology among CMV strains (58,142) it should be possible to develop a probe which will hybridize to all HCMV. In addition the low amount of homology between the genomes of CMV and the other herpes viruses such as HSV-1, HSV-2 and EBV indicates that hybridization could be expected to be specific for CMV.

The goal of this project has been to select a cloned fragment of the CMV genome of strain AD169 (109) which is common to all strains of the virus but unique to CMV. Such a fragment could then be used as a labelled probe for detection of CMV at an early stage of infection in tissue culture or directly in clinical specimens. The large size of the viral DNA (150×10^6 daltons) precludes the cloning of the entire genome, thus an appropriate fragment needs to be selected from among those produced by restriction endonuclease digestion of the viral DNA.

Two potential problems must be considered in the selection of a cloned fragment. First, in spite of the large degree of homology among isolates of CMV, there is still significant lack of homology between particular segments of the genomes of individual strains. La Femina et al. (74) showed that CMV Davis and AD169 have 2000-4000 base pair deletions relative to CMV Towne. The deletions appear to occur at the extreme internal segments of the IR_L . The rest of the genomes of CMV Towne and Davis show a high degree of homology. Weststrate et al. (142) reported a

similar study comparing CMV AD169 and CMV SG. The majority of the viral DNA of the two strains cross-hybridized, but there were two areas which did not. These sequences were in the repeats and at the left end of the U_L segment. In selecting a fragment for a CMV cross-reactive probe, therefore, it would be best to avoid fragments which may not contain sequences capable of hybridizing to all samples of HCMV DNA.

The second problem is that of homology between portions of the CMV genome and mammalian cellular DNA sequences. The segments of the CMV DNA which are homologous to portions of the v-myc and c-myc genes (14, 41, 85, 116) and those which hybridize to intermediate repetitive cell DNA sequences (93, 98) could lead to an undesirable background level of hybridization of the probe in tissue culture. Restriction endonuclease maps are available for determining which fragments may contain sequences that might increase the level of background hybridization (21, 49, 75, 88).

To develop a probe for the purposes of this project CMV AD169 DNA was isolated from extracellular virus in tissue culture fluid. The DNA was digested with BamHI and fragments were inserted into pBR322. The plasmids were transformed into E. coli SF8 and screened for tetracycline sensitivity. Recombinant clones were analyzed for large inserts. An insert co-migrating with the CMV BamHI A/B fragments was selected and subsequently identified as the 15 kb B fragment. The recombinant plasmid was radiolabelled with 32 P-deoxycytidine by nick translation and assayed for cross-reactivity with restriction endonuclease digests of EBV, HSV-1 and HSV-2 (80, 114). The specificity of the labelled probe was demonstrated by hybridization to cells in tissue culture infected with different CMV strains from clinical sources. Sensitivity of the radiolabelled

probe was determined by hybridization to dilutions of CMV AD169 DNA and cell-free virus stocks. Patient urine specimens submitted for CMV culture were used to demonstrate the potential clinical application of the probe for CMV detection or identification.

A final phase of this project has been a comparison of the sensitivity of the probe labelled with ^{32}P with the same probe labelled with biotin. There are two potential advantages of a biotin-labelled probe for clinical laboratory application: 1) elimination of the need for a radioactive isotope and 2) stability of the probe activity.

The Enzo-Biochem commercial nick translation kit was used to incorporate biotinylated deoxyuridine into the probe. The detection system is based on the strong binding of the protein avidin to biotin. Avidin is tagged with horseradish peroxidase and addition of the enzyme substrate produces a colored product at the site of binding to the probe (4,8,80).

The biotin-labelled probe was hybridized to CMV AD169 DNA, stock virus dilutions and cells infected with different CMV strains. The results of these hybridizations were compared to the parallel results obtained under the same conditions with the radiolabelled probe.

MATERIALS AND METHODS

Virus. CMV strain AD169 (109) was obtained from Dr. Marc Beem at the University of Chicago. A second sample of CMV AD169 was kindly donated by Dr. Robert Betts at the University of Rochester.

Virus stocks were frozen and maintained at -70°C in medium containing 10% dimethylsulfoxide (DMSO) and 10% fetal calf serum (FCS). When needed the stock virus was rapidly thawed in a 35°C incubator and diluted appropriately in Earles balanced salt solution (EBSS). Samples of CMV isolated from patient specimens received by the clinical microbiology laboratory at Loyola University Medical Center were also collected, passaged and stored.

Cells. Human foreskin fibroblasts (HFS HR218 passage 19) were purchased from HEM Research, Rockville, MD for large-scale growth of the virus. Additional HFS (MRHF passage 22-23) were purchased from M.A. Bioproducts, Walkersville, MD. These cells were routinely passaged 1:2 up to a maximum passage of 29. In some experiments human embryonic lung (HEL MRC-5) cells also from M.A. Bioproducts or WI-38 cells (Flow Laboratories, McLean, VA) were used.

For storage cells were frozen and maintained under liquid nitrogen in medium containing 10% DMSO and 10% FCS. When needed the cells were quickly thawed in a 35°C incubator, placed in 10 ml of EBSS and centrifuged at 1500 rpm in a Sorvall H4000 rotor at 4°C for 15 min. The supernatant was discarded and the cell pellet was resuspended in 5 ml of growth medium (see below). The suspension was then plated in the appropriate number of flasks or plates for a 1:2 split.

Confluent monolayers were detached with filtered (0.22 μ m Millipore) 0.25% trypsin. Generally incubation at 35°C for 10 min was sufficient for the trypsin to remove the cells; however, monolayers which had been under maintenance medium (see below) at confluency for several days required longer incubation times. Detached cells, diluted 1:4 in trypan blue, were counted with a hemocytometer.

Tissue Culture Media. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% FCS, 7.5% NaHCO_3 , (to adjust pH to 7.2), L-glutamine (1 mM), HEPES buffer (20 mM), gentamicin (50 μ g/ml) and amphotericin B (2.5 μ g/ml). This is referred to as growth medium. Maintenance medium was Eagle's MEM with the same supplements except only 1% FCS and no HEPES. Earles balanced salt solution was used for washing cell monolayers and for making serial dilutions as virus. The overlay for plaque assays consisted of equal parts of 0.6% agarose and double strength maintenance medium (140).

Bacteriological Media. L broth for growth of E. coli consisted of tryptone (10g), yeast extract (5g) and NaCl (5g) in a total volume of 1 liter. The pH was adjusted to 7.1-7.4 with NaOH (80).

For large-scale isolation of plasmid DNA the bacteria were grown in A.B. minimal medium consisting of K_2HPO_4 (3g/l), NH_4Cl (1g/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.3 g/l), KCl (0.15 g/l), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.01 g/l), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.5 mg/l), glucose (5 g/l), Casamino acids (0.5%), L-tryptophan (25 μ g/ml) and thiamine (5 μ g/l) (11).

For isolation and maintenance of recombinant clones nutrient agar plates (1.5% agar, 0.8% BBL or Difco nutrient broth base) were used. The medium was supplemented with 100 μ g/ml ampicillin or 10 μ g/ml tetracycline.

Reagents and Chemicals. The following is a list of sources for the commonly used reagents and chemicals: FCS and amphotericin B (Fungizone) from Gibco, Grand Island, NY; EBSS, Eagle's MEM, L-glutamine, NaHCO_3 , HEPES buffer and trypsin from Flow Laboratories, McLean, VA; ^3H -thymidine and ^{32}P -deoxycytidine from New England Nuclear; gentamicin (Garamycin) from M.A. Bioproducts; CsCl from Harshaw Chemical, Solon, OH; Pronase B from Calbiochem-Behring, LaJolla, CA; Sarkosyl, sodium dodecyl sulfate (SDS), Trizma base, disodium EDTA, bovine serum albumin (BSA), dithiothreitol (DTT), calf thymus DNA, lysozyme and RNase A from Sigma; restriction endonucleases BamHI, HindIII, PstI, EcoRI and BglII from BRL, Gaithersburg, MD; boric acid, sodium citrate, sodium chloride, formamide from Mallinkrodt, St. Louis, MO; polyvinylpyrrolidone (PVP), ficoll 400, dextran sulfate and Sephadex G-50 from Pharmacia, Uppsala, Sweden.

Plaque Assay. The method of Wentworth and French (140) was modified for titering and plaque purifying the virus. HFS cells were propagated in growth medium in 24-well (diameter 16 mm) tissue culture plates (Falcon 3047) until just subconfluent (at least 2-3 days) (18). Virus dilutions from 10^{-1} to 10^{-6} were made in EBSS. The growth medium was removed from each well and replaced with 0.1 ml of viral inoculum. Final viral dilutions were 10^{-2} to 10^{-7} . The virus was allowed to adsorb for 2 h at 35°C . The excess liquid was removed at the end of the adsorption period and replaced with an overlay of equal parts 0.6% agarose and double strength maintenance medium. Seven days later a second overlay was poured on top of the first. Plates could then be held for as long as 4-6 weeks without drying out.

Plaques were counted in wells containing no more than 10-15 well-isolated plaques. The titer in plaque-forming units (pfu) was calculated according to the dilution. Wells containing no more than three well-isolated plaques were selected for plaque purification. A capillary pipette was used to withdraw medium overlying well-developed plaques. This inoculum was then placed on HFS cell monolayers in 25 cm² flasks (Corning) for further culture.

Passage of Stock Virus. Maintenance medium was changed every 5-7 days until CPE reached 80-90%. From 3-4 days after 100% CPE was evident a 10⁻⁵ dilution of the extracellular fluid was made in EBSS. A 0.1 ml sample of the dilution was used to inoculate monolayers in 25 cm² tissue culture flasks. The MOI usually was low enough to maintain high-titer virus (126).

Electron Microscopy. Electron microscopy was performed by Dr. Raoul Fresco, at the Loyola University Medical Center, on a preparation of cells infected with 2 different samples of strain AD169. The first was a three-times plaque purified sample from the strain obtained from Dr. Marc Beem. The other was a one-time plaque purified sample of the strain supplied by Dr. Robert Betts.

Large-Scale Growth of Plaque-Purified Stock Virus. In earlier experiments 24 tissue culture flasks (150 cm²) of HFS cell monolayers at passage 25-27 were inoculated with 1 ml each of a 1:1,000 dilution of virus stock supernatant. The MOI was between 0.01 and 0.001. The virus was allowed to adsorb for 2 h and then 35 ml of maintenance medium was placed in each flask. After 7-8 days each flask was labelled with ³H-thymidine (3 µCi/ml). Extracellular virus was harvested at 13-15 days

post-infection, which was 3-4 days after 100% CPE was reached in all flasks.

In later experiments to increase the yield of virus, the cells were initially propagated in tissue culture flasks (150 cm^2), and then split to 10 roller bottles (850 cm^2). Each roller bottle was inoculated at subconfluency with 5 ml of a plaque-purified virus stock dilution. The MOI was between 0.1 and 0.01. Each roller bottle contained 125 ml of maintenance medium, which was changed at 7 days. The supernatant fluid was harvested 3 days after 100% CPE was observed.

Purification of Virus From Supernatant Fluid. Several changes in this procedure were made from experiment to experiment to increase the yield of DNA. In earlier experiments the clarified supernatant fluid was centrifuged in a Beckman Ti 45 rotor at 25,000 rpm for 1 h through a 20 ml cushion of 20% (w/v) D-sorbitol in 0.05 M Tris-hydrochloride (Tris-HCl), pH 7.2, containing 0.001 M MgCl_2 and 100 μg bacitracin per ml (124, 126). The pellets were resuspended by vortexing in a total of 1 ml Tris-buffered saline (TBS 0.05 M Tris-HCl pH 7.2, 0.15 M NaCl) and combined. An additional 1 ml of TBS was used to sequentially rinse out the tubes. This was added to the combined pellet suspension. This suspension was layered onto a discontinuous sorbitol gradient (75%/48%/20% w/v). The gradient was centrifuged at 26,500 rpm at 20°C for 60 min in a Beckman SW 28 rotor (130). The visible band at the interface of 75%/48% sorbitol was collected by side puncture. This material was diluted with TBS to 38 ml total volume and repelleted at 26,500 rpm at 20°C for 3 h in the SW 28 rotor.

In later experiments 5% polyethylene glycol (PEG) 6000 (34,52) was

added to the supernatant fluid to precipitate the virus. Once the PEG was dissolved the fluid was held at 4°C for 4 h and then centrifuged at 7,000 rpm for 30 min at 4°C in a Beckman JA-14 rotor. The supernatant fluid was poured off and the viral pellet was resuspended in TBS. The virus suspension was placed on a discontinuous sorbitol gradient and purified as described above.

The difficulty in resuspending PEG-precipitated virus prompted a further modification of the pelleting procedure (65,75). The clarified supernatant fluid was centrifuged for 90 min at 12,000 rpm in a Sorvall GSA rotor. The supernatant fluid was poured off and the remaining viral pellets were drained and resuspended in a total volume of 2 ml of buffer (0.4 M NaCl, 5 mM Tris-HCl pH 8.0, 100 mM EDTA). An additional 1 ml of buffer was used to wash the centrifuge bottles to collect any residual virus.

Extraction of Viral DNA. In earlier experiments the final viral pellet was resuspended in 2 ml of buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.01 M EDTA pH 8.0) (130). SDS was added to a final concentration of 1% followed by RNase A (50 µg/ml) and pronase (1 mg/ml). This solution was incubated for 1 h at 37°C then extracted twice with 2 volumes phenol-chloroform-isoamyl alcohol (50:48:2) and once with 2 volumes of chloroform-isoamyl alcohol (24:1).

The treated lysate was placed in CsCl at a density of 1.72 g/ml and centrifuged at 35,000 rpm at 20°C for 48 h in a Beckman type 50 rotor. The resulting gradient was fractionated; counts per minute (cpm) and refractive index for each fraction were determined. Fractions showing peak radioactivity and corresponding to the density of CMV DNA (1.716 g/ml)

were pooled and dialyzed overnight with three changes of buffer (5 mM Tris-HCl, 1 mM EDTA pH 8.0).

In later experiments a modification of the method of La Femina and Hayward (75) was used. RNase A (20 µg/ml) was added to the virus suspension, which was then incubated at 37°C for 15 min. Sarkosyl (2%) and SDS (0.5%) were added, and the lysate was incubated at 37°C for 60 min. Pronase (1 mg/ml) was added and incubation at 37°C was continued for an additional 2 h. After extraction with phenol followed by chloroform/isomyl alcohol (98:2), the aqueous layer was dialyzed as follows: 1 h against 2 L 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 300 mM NaCl; overnight against 2 L 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0; 1 h in another 2 L of the same buffer.

In addition to the extraction of DNA from extracellular virions, the remaining adherent cells were trypsinized, washed and pelleted. The cells were resuspended in a small volume of growth medium with 10% DMSO and frozen at -70°C for future extraction of intracellular virion DNA.

Culture for Infectivity During Purification of Virus. Aliquots (0.1 ml) from the original pellets, sorbitol gradient and repelleted virions were cultured to determine the amount of viral infectivity remaining at each step. A plaque assay was performed on each aliquot according to the procedure outlined above (140). Titers expressed as pfu were determined after 21 days.

Concentration of DNA. DNA samples were precipitated with 1/10 volume 3 M potassium acetate plus 2 volumes of 95% ethanol. The samples were then placed on dry ice for 20 min and centrifuged in a Fisher model 235 microfuge for 10 min. The supernatant was poured off and the DNA

precipitates were resuspended in a small volume of 10 mM Tris-HCl, 0.1 mM EDTA pH 8.0. The DNA was stored at 4°C or frozen at -20°C.

Isolation of Plasmid pBR322. One liter of L broth containing 5 µg/ml tetracycline was inoculated with 10 ml of a 50 ml overnight preculture of E. coli strain RR1. The organism was grown with shaking in a 35°C incubator (17) to a reading of 100 on a Klett-Summerson model 800-3 colorimeter (No. 66 red filter). The bacteria were pelleted at 8,000 rpm for 5 min in a Sorvall GSA rotor. The supernatant was discarded, and the pellets were resuspended in ice-cold TE buffer (0.05 M Tris-HCl, 0.05 M EDTA pH 8.0) and repelleted a second time. The pellets were then resuspended in 0.05 M Tris-HCl, 0.05 M EDTA pH 8.0 containing 15% sucrose and 1 mg/ml lysozyme. This suspension was incubated at room temperature for 10 min followed by the addition of 0.1% Triton X-100 in 0.05 M Tris-HCl, 0.05 M EDTA pH 8.0. The lysate suspension was incubated at 37°C for 30 min followed by short incubations at 55°C until lysis appeared complete. The lysates were centrifuged at 19,000 rpm for 1 h at 4°C in a Sorvall SS-34 rotor. The non-viscous supernatant was removed and added to CsCl containing 0.1 mg/ml ethidium bromide for a final density of 1.59 g/cm³. The sample was centrifuged at 38,000 rpm for 38 h in a Beckman VTi 50 rotor.

The lower u-v fluorescent band in the gradient was collected. The ethidium bromide was removed from the collected material by repeated extraction with isopropanol saturated with 20X SSC (1X SSC is 0.15 M NaCl, 0.015 M trisodium citrate). The plasmid DNA sample was dialyzed overnight against 10 mM Tris-HCl, 0.1 mM EDTA pH 8.0. The dialysate was precipitated with ethanol as outlined above. A 10 µl aliquot of the plasmid

sample was subjected to agarose gel electrophoresis directly. Another aliquot of the sample was digested with restriction enzymes HindIII and HinfI, and the fragments were analyzed by agarose gel electrophoresis.

Cloning Procedure. Approximately 1 μ g of CMV DNA and 0.1 μ g of the vector pBR322 were digested separately with BamHI. The digests were ethanol precipitated and resuspended in ligase buffer (60 mM Tris-HCl pH 8.0, 33 mM NaCl, 10 mM MgCl_2) containing ATP (0.5 mM) and DTT (5 mM). The ligation reaction mixture contained the precipitated digests of insert (CMV) DNA and vector (pBR322) DNA at a concentration ratio of 10:1 and 1 unit of T_4 ligase. A control reaction mixture contained only digested vector DNA with the same reagents. Both ligation and control mixtures were incubated overnight at 14°C. Following incubation the reaction mixtures were again precipitated with ethanol and resuspended in 60 μ l of 0.1 M Tris-HCl pH 7.2. Aliquots of the concentrated mixtures were subjected to agarose gel electrophoresis for analysis.

The streptomycin-resistant E. coli strain SF8 (genotype: hsdR⁻, hsdM⁻, recB, recC, lop-11 (ligase overproducer supE44), gal-96, leuB6, thi-1, thr; a gift from Dr. Masajasur Nomura, University of Wisconsin) was grown in an overnight preculture in 5 ml of L broth. A 0.2 ml sample of this preculture was added to 20 ml of L broth and grown to a Klett reading of 100. Transformation of the ligation reaction mixture, the control mixture and a standard pBR322 untreated control was performed according to the method outlined in Davis et al. (17). The bacteria were sedimented by centrifugation at 5,000 rpm in a Sorvall SS-34 rotor for 5 min at 2°C. The pelleted cells were resuspended in 10 ml cold 50 mM CaCl_2 and incubated for 5-60 min at 0°C. The centrifugation

and resuspension steps were repeated in a 1 ml volume of CaCl_2 . The bacteria were then added in a 2:1 (v/v) ratio to the ligation mixture, the control mixture and the untreated pBR322 control and allowed to sit for 10 min at 0°C . Each sample was incubated for 2 min at 37°C followed by 10 min at 25°C . Ten volumes of L broth were added to each sample and they were again incubated at 37°C for 20 min. Dilutions of the cells from each sample were then plated out on nutrient agar plates containing ampicillin (100 $\mu\text{g/ml}$). Colonies which appeared on the plates from the ligation mixture transformation were numbered and picked to nutrient agar plates containing either ampicillin (100 $\mu\text{g/ml}$) or tetracycline (10 $\mu\text{g/ml}$). Colonies which were resistant to ampicillin but sensitive to tetracycline were selected for further plasmid analysis.

Rapid Technique for Plasmid Analysis. Recombinants from the cloning procedure were analyzed by the method of Holmes and Quigley (55). The organisms were grown overnight in 1.5 ml of L broth. Half of the cell suspension was transferred to a 1.5 ml microfuge tube and pelleted for 3 min at 4°C in a Fisher model 235 microfuge. The supernatant fluid was removed and the pellets were resuspended in 100 μl STET buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 8% sucrose, 5% Triton X-100). Eight microliters of a 10 mg/ml solution of lysozyme was added and the tubes were placed in boiling water for 40 sec. The tubes were recentrifuged for 10 min at 4°C and the supernatant was precipitated with ethanol at -18°C for 5 min. After another 5 min centrifugation the precipitates were resuspended in water at room temperature. Aliquots of these samples were then subjected directly to agarose gel electrophoresis. For restriction enzyme digestion analysis the samples were precipitated a second time

with ethanol and treated with RNase A for 30 min at 37°C.

Agarose Gel Electrophoresis. Electrophoresis of undigested viral or plasmid DNA was performed on 0.7% horizontal agarose gels in a buffer (pH 8.0) containing 89 mM Trizma base, 2.5 mM EDTA, 89 mM boric acid with a constant current of 50 mA for 3-4 h. Bacteriophage lambda DNA standards ranging from 0.125 µg to 1.0 µg were placed on the same gels for quantitation.

For analysis of restriction enzyme digests the samples were electrophoresed at a constant current of 17-19 mA for 18-20 h. HindIII digests of lambda DNA were run as molecular weight standards on the same gels. Following electrophoresis all gels were stained with ethidium bromide (0.5 µg/ml), placed on a u-v transilluminator and photographed with Polaroid type 55 film.

Large-Scale Isolation of Recombinant Plasmid DNA. Ten milliliters of a 50 ml overnight preculture was used to inoculate 1 L of A.B. minimal medium containing no antibiotics. The organism was grown with shaking at 35°C. When a Klett reading of 90-100 was reached, chloramphenicol (250 µg/ml) was added to the culture medium for plasmid amplification. Incubation was continued for 14-16 h at which time the cells were harvested. The plasmid DNA was isolated as outlined above for pBR322 (17).

Restriction Endonuclease Digestion. Single digests of CMV or plasmid DNA were performed with the restriction enzymes BamHI, EcoRI, HindIII, BglII or PstI. The buffer used for all digests contained 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂ and 50 mM NaCl and 1 mM DTT. The reaction mixtures were incubated at 37°C for 60 min except for those containing PstI, which

were incubated at 30°C. The reactions were stopped by heating at 65°C for 5 min.

For double digests the DNA was first digested with BamHI for 60 min at 37°C. The salt concentration was adjusted where necessary; the second enzyme was added, and incubation was continued for another 20 min at 37°C except for the PstI digests, which were incubated at 30°C. Reactions were again stopped by heating the samples at 65°C for 5 min. In later experiments both enzymes were added together at the beginning of the double digestion and allowed to react for a total of 60 min in the buffer described above.

Southern Blotting. Identification of selected cloned fragments was carried out by standard hybridization techniques (80,114). CMV DNA was digested with BamHI, EcoRI or HindIII and subjected to agarose gel electrophoresis as outlined above, and the gel was stained with ethidium bromide for 1 h. The DNA was nicked by exposing the gel for 4 min to u-v light using a transilluminator. The gel was destained with distilled H₂O for 30 min, washed with gentle agitation two times for 15 min each with 0.5 M NaOH/1.5 M NaCl and neutralized by washing two times for 20 min each with 0.5 M Tris-HCl pH 7.5/1.5 M NaCl. The DNA was then transferred from the gel to a nitrocellulose filter overnight using a BRL blotting apparatus with 10X SSPE buffer (1.8 M NaCl, 100 mM NaH₂PO₄·H₂O, 10 mM EDTA). At the end of the transfer the gel was restained with ethidium bromide and photographed to determine how much DNA remained. The filter was baked in a vacuum oven at 80°C for 2 h and then placed in a heat-seal bag.

Nick Translation. Nick translation (106) of the probe DNA was per-

formed using a nick translation kit (Amersham). The probe reaction mixture contained 1-2 μg probe DNA, 20 μl buffer solution, 1-2 μl (α - ^{32}P)-dCTP (10-20 μCi) and 10 μl of enzyme solution in a volume of 100 μl . The reaction was allowed to proceed at 15°C for 90 min at which time it was stopped by the addition of 100 μl of 0.02 M EDTA containing 0.2% SDS. The probe mixture was loaded on a 10 ml Sephadex G-50 column equilibrated in 50 mM Tris-HCl, 10 mM EDTA. Progress down the column was monitored with a Geiger counter. Approximately 1.5 ml of buffer containing the probe was collected after the predetermined void volume. The probe was denatured by heating to 95°C for 10 min then quick-cooled on ice.

Hybridization. Prehybridization of nitrocellulose filters was carried out in heat-seal bags containing a solution of 5X SSPE, 0.3% SDS and 100 $\mu\text{g}/\text{ml}$ denatured calf thymus DNA. Approximately 30 ml of the prehybridization fluid was added for a 20 x 20 cm filter, and the sealed bag was incubated at 65°C for at least 1 h.

The GeneScreen Plus membranes (New England Nuclear) were prehybridized according to the manufacturer's protocol in a solution containing 0.2% PVP, 0.2% ficoll 400, 0.2% BSA, 0.05 M Tris-HCl pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate and 100 $\mu\text{g}/\text{ml}$ calf thymus DNA. The prehybridization was carried out in a heat-seal bag at 65°C for 6 h with constant agitation.

Half of the probe sample was added to the nitrocellulose filters in fresh hybridization fluid in the heat-seal bag. Hybridization was carried out at 65°C overnight. The filter was removed and washed with gentle agitation four times for 30 min each at 45°C in 100-150 ml 2X SSPE containing 0.2% SDS. The filter was dried for 2 h at room temperature

and then placed between two pieces of Kodak X-Omat AR X-ray film in a cassette with Cronex Lightning Plus intensifier screens (Dupont). The cassette was held in a -70°C freezer for 19-20 h.

For the GeneScreen Plus membrane hybridizations the denatured probe was added to the prehybridization fluid along with 100 $\mu\text{g}/\text{ml}$ denatured calf thymus DNA. The membranes were incubated with agitation for 36-48 h.

The washing procedure for the GeneScreen Plus membranes was as follows: twice with 100 ml 2X SSC at room temperature for 5 min each; twice with 200 ml 2X SSC/1% SDS at 65°C for 30 min each; twice with 100 ml 0.1X SSC at room temperature for 30 min each. Membranes were air-dried and placed in an X-ray cassette as previously described.

Preparation of Filters for Direct Cell or Virus Hybridization.

MRC-5 cells grown in 16 x 125 mm tubes or 25 cm^2 flasks were infected with CMV AD169 or clinical CMV isolates. The inoculum consisted of different dilutions of the virus, and infection was allowed to proceed for various periods of time. The cells were harvested by trypsinization, washed in 1X EBSS, pelleted at 1500 rpm for 15 min in a Beckman Model TJ-6 centrifuge and resuspended in 100-200 μl of EBSS. Appropriate dilutions of the cell suspensions were also made. Nitrocellulose or GeneScreen Plus membranes were spotted with 5 μl of the cell suspensions or dilutions and allowed to air dry. The membranes were processed by placing them on top of strips of Whatman 3MM chromatography paper soaked in the following solutions as described by Brandsma and Miller (7): 0.5 M NaOH for 7 min; 0.6 M NaCl/0.5 M Tris-HCl pH 6.8 twice for 1 min each; 1.5 M NaCl/0.5 M Tris-HCl pH 7.5 for 5 min. The membranes were air-dried

for 20 min, floated on 95% ethanol and again air-dried for 5 min. They were then washed twice in chloroform, air-dried for 15 min and washed with 0.3 M NaCl. The nitrocellulose filters were baked in a vacuum oven at 80°C for 3 h; the GeneScreen Plus membranes required no baking.

Extracellular samples of the different virus strains or purified viral DNA were also spotted directly on GeneScreen Plus membranes. To place more than 20 µl of the sample in a 2 cm² area it was necessary to spot 10 µl aliquots and allow the filter to dry. Additional 10 µl aliquots could then be spotted over the same area as long as complete drying occurred between applications. These membranes were prepared for hybridization in the same manner as those spotted with cells.

In later experiments a Minifold (Schleicher & Schuell, Keene, NH) dot blot filtration apparatus was used to prepare GeneScreen Plus membranes for direct hybridization of the probe to infected cells or cell-free virus DNA. The membrane was prepared by suctioning 1X SSC through the wells. Sample volumes up to 0.5 ml could then be placed in the appropriate wells and filtered under slight vacuum.

Processing of Clinical Specimens. Ten milliliter samples of urine specimens submitted for CMV culture were collected and stored at -70°C in a 15 ml centrifuge tube for simultaneous analysis. The samples were thawed at 55°C. The urines were centrifuged at 3,000 rpm for 5 min in a Beckman TJ-6 centrifuge, and the supernatants were transferred to 15 ml Corex tubes (Corning). The samples were centrifuged at 14,000 rpm for 90 min at 4°C in a Sorvall SS34 rotor. The supernatant was discarded; the pellet was treated with 0.25 ml of 0.3 M NaCl/0.5 M NaOH; the samples were vortexed briefly and allowed to stand for 15 min at

room temperature. An equal amount of 1 M sodium acetate was added. The entire sample was placed in a well of the Minifold filtration apparatus and drawn through the GeneScreen Plus membrane with a slow vacuum. The membranes were then treated according to the above procedure for spotting of infected cells or cell-free virus. The hybridization procedure was also the same as previously described. In addition to autoradiograms of the membrane, scintillation counting was performed on specimen squares cut from the membrane.

Nick Translation of Biotinylated Probe DNA. Nick translation of pNSL225 was carried out according to the Enzo-Biochem protocol for the nick translation kit. Tritiated dATP (12 μ l) was lyophilized in a 1.5 ml centrifuge tube to remove the ethanol. Following lyophilization the kit reagents were added in order: 5 μ l nick translation buffer, 5 μ l nucleoside triphosphate solution, 5 μ l biotinylated dUTP, 1 μ g DNA, 4 μ l DNase I (appropriately diluted), 4 μ l DNA polymerase I, water for a final volume of 50 μ l. The reaction mixture was placed in a 14°C water bath for 2 h. To stop the reaction 5 μ l of the stop buffer (0.2 M EDTA) was added.

The biotinylated probe was separated from unincorporated nucleotides on a 3.5 ml Sephadex G-50 column equilibrated with 10 mM Tris-HCl pH 7.5, 1 mM EDTA. Five-drop fractions were collected; 2 μ l aliquots of each fraction were counted by liquid scintillation. Fractions forming the first peak of radioactivity were collected and pooled.

Alternatively the reaction mixture was placed on a spin column. The spin column was constructed as described in the Enzo-Biochem protocol using a 1 ml tuberculin syringe filled with Sephadex G-50 equili-

brated with 10 mM Tris HCl, 1 mM EDTA and placed inside a 15 ml conical centrifuge tube. The column was packed by low-speed centrifugation (1500 rpm for 4 min in a Beckman TJ-6 centrifuge). A 1.5 ml Eppendorf tube was used to collect the probe material which passed through the column following low-speed centrifugation under the exact conditions used to pack the column.

Hybridization and Detection of Biotinylated Probe. Prehybridization of spotted membranes was carried out according to the GeneScreen Plus manufacturer's protocol as described above. The hybridization solution contained: 0.02% PVP, 0.02% ficoll 400, 0.02% BSA, 5X SSC, 50% formamide, 20 mM Na phosphate buffer pH 7.0, 10% dextran sulfate, 0.1% SDS, 1 mg/ml freshly boiled calf thymus DNA, 200-500 ng/ml freshly boiled probe DNA. The membranes with the hybridization fluid were placed in a heat-seal bag and incubated with shaking at 42°C for 24-48 h. At the end of this period the membranes were washed as follows: twice for 15 min each in 2X SSC/0.1% SDS at room temperature; twice for 15 min each in the same solution at 65°C; once in 2X SSC alone for 15 min at room temperature.

The washed membranes were blocked in a dish containing a solution of 2% BSA and 0.1% Triton X-100 in 10X PBS for 1 h at 37°C. The horseradish peroxidase complex (Detek I-hrp) was diluted in buffer as provided by the detection kit, added to the membranes in a heat-seal bag and incubated for 30 min at 37°C. The membranes were then removed from the bag and washed as follows: 3 times for 5 min each in 0.5 M NaCl, 10 mM phosphate buffer pH 6.5. 0.1% BSA, Tween 20 (0.5 ml/l) at room temperature; 2 times with 2X SSC, 0.1% BSA, Tween 20 (0.5 ml/l).

The enzyme substrate was prepared by adding 10 ml of 10 mM Tris-HCl pH 7.5 to 2.5 mg of diaminobenzidine tetrahydrochloride (DAB). Cobalt chloride (100 μ l of a 1% solution) was added to the DAB, which was then placed on ice for 10 min in the dark. Finally 7.5 μ l of 30% H_2O_2 was added, and the solution was applied to the membrane in a heat-seal bag. The bag was placed in the dark for 10 min for full color development. After this time the membrane was washed with 2X SSC and examined for probe hybridization.

RESULTS

Growth Characteristics, Titer and Plaque Purification of CMV.

Samples of CMV AD169 could be passaged in culture from the extracellular tissue culture fluid once the CPE had reached approximately 50%. Higher extracellular titer, however, occurred 2-4 days after 100% CPE was reached (57,124). Attempts to passage cell-free fluid with less than 50% CPE were routinely unsuccessful, although passage of infected cells to new monolayers was possible whenever there was detectable CPE.

Three clinical isolates (LU-1, LU-2, LU-3) were originally highly cell-associated. In infected monolayers the CPE remained less than 25% even after weeks of culture. Monolayers were repeatedly passaged when CPE had been visible for 1-2 weeks. After approximately 4-5 passages of infected monolayers over a period of several months, the virus became less cell-associated. CPE progressed to 100% of the monolayers, and passage of the tissue culture fluid alone was sufficient to infect new monolayers. Continued passage of the supernatant fluid of these strains resulted in a further increase in the titer of the extracellular fluid (Table 1).

For all strains of CMV it was necessary to passage the virus at a low MOI (less than 1) to achieve a high extracellular virus titer. The number of defective particles increases when the MOI is greater than one, which means the pfu to particle ratio decreases (16,112, Table 1).

The titers were determined by the plaque assay method of Wentworth and French (140) except that 24-well plates were used instead of 60 mm

TABLE 1

Titers of CMV strains after passage.

Isolate Number	Original Titer (pfu/ml)	Titer after Passage (pfu/ml)	MOI
AD-169	1×10^6	2×10^2	>1
AD-169	$1 \times 10^6 - 1 \times 10^7$	$1 \times 10^6 - 1 \times 10^7$	0.01-0.001
LU-1	2×10^5	4.5×10^7	0.01-0.001
LU-2	1×10^4	1×10^6	0.01-0.001
LU-3	1×10^5	1.5×10^6	0.01-0.001

dishes. This change made it possible to inoculate a larger number of replicate cultures with fewer cells for each assay. One other modification involved plating the cells well below confluency and allowing them to grow for 24-48 h to slight subconfluency. The cells were then much more adherent and less easily disrupted by the virus inoculation and subsequent agarose overlay. For the isolates of AD169 and the clinical strains LU-1, LU-2, LU-3, the highest extracellular titer observed was 4.5×10^7 (Table 1). Depending on the passage and growth conditions the titers usually ranged between 1×10^6 and 1×10^7 , which is lower than the optimal titer of 5×10^8 reported for the CMV Towne strain (75). The use of roller bottles, however, increased the titer approximately ten-fold (Table 2).

The plaque assays were also used for plaque purification. By selecting wells in which there were fewer than 5 plaques it was possible to pick isolated plaques for passage to 25 cm^2 flasks. It was necessary, however, to wait until there was some clearing of the cells in an infected focus to assure the presence of extracellular virus in the overlay medium. This usually required 5-7 days from the initial appearance of the focus. Since the plaques were selected from the highest dilutions, the time from inoculation to the appearance of plaques in these wells was generally 12-14 days. With additional time to clearing of the plaque the total time required for plaque selection was about 3 weeks. The plaque-purified inoculum was then passaged to HFS cells in 25 cm^2 flasks at an MOI between 0.01 and 0.001 to propagate the virus for further plaque purification or for freezing for stock samples. Aliquots of the final plaque-purified samples were examined by electron microscopy

TABLE 2

Effect of culture vessel on titer of CMV AD169.

Experiment Number	Culture Vessel	Titer of Supernatant (pfu/ml)	Total Virus Yield (pfu)
1	24 flasks (150 cm ²)	2×10^7	1.7×10^{10}
2	24 flasks (150 cm ²)	1×10^7	8.4×10^9
3	10 roller bottles (850 cm ²)	4×10^8	5.0×10^{11}

for morphological confirmation, that the virus was CMV.

To obtain a stock of AD169 for large-scale isolation of DNA the original sample was plaque-purified three times. The final stock virus had a titer of 2.5×10^7 . To maintain the stock a 1×10^{-6} dilution of the plaque-purified virus was passaged in 25 cm^2 flasks. When the MOI was appropriately low, CPE did not appear for at least 7 days after inoculation. A very few discrete foci of infection appeared at that time, and these progressed to 100% CPE by 12-14 days. The extracellular fluid was ready for harvest 3-4 days later.

Large-Scale Growth and Purification of Virions. Several modifications were made in this procedure because there was difficulty in obtaining sufficient quantity of virus for DNA extraction. In the earliest experiments the virus was propagated in 24 tissue culture flasks (150 cm^2), which yielded about 850 ml of culture fluid and titers of approximately 1×10^7 pfu/ml. To increase the total virus yield 850 cm^2 roller bottles were used. The total surface area of the inoculated monolayers was more than twice the total area of the 150 cm^2 flasks, and at the same time the total tissue culture fluid volume required to maintain the roller bottle cultures was only 1.5 times greater than the volume for the flasks (1200 ml versus 850 ml).

In all purification procedures attempted the supernatant fluid was first cleared of cell debris by low-speed centrifugation. In the earliest experiments the virus was pelleted by ultracentrifugation through a sorbitol cushion (124,126), which allows the best preservation of infectivity. A modification which was introduced later was the use of 5% PEG 6000 to precipitate the virus from the supernatant fluid fol-

lowed by low-speed centrifugation to pellet the virus (34,52). There were visible viral pellets obtained with both of these methods, but the total yield was still insufficient for adequate DNA extraction (Table 3).

Further purification steps in both of the above procedures involved the use of a discontinuous CsCl gradient (124) or a discontinuous sorbitol gradient (130). Both of these manipulations appeared also to decrease the final virion yield, although visible bands appeared at the appropriate interfaces (Table 3).

To follow the virus through these purification steps the original cultures were labelled with ^3H -thymidine. In addition the infectivity of the material obtained after each step was tested. In most of the experiments there was significant radioactivity and infectivity associated with the collected samples.

The problems encountered in the earlier attempts to purify the virus seemed to involve either insufficient initial viral titer or loss of virions during purification. To simplify the procedure the clarified supernatant fluid from 10 roller bottles was directly pelleted at 23,430 x g without a sorbitol cushion or PEG precipitation (75). This modification led to the successful collection of a sufficient quantity of virus for DNA extraction.

Extraction of DNA. The procedure published by Stinski et al.

(124,126) was followed for the first attempts to extract viral DNA. The virions were lysed and digested with Sarkosyl, SDS and proteinase K. The lysate was added to CsCl with ethidium bromide (50 $\mu\text{g}/\text{ml}$) at a density of 1.56 g/cm^3 . At equilibrium the ethidium bromide was present as a red band at the interface of the CsCl solution and the mineral oil.

Table 3. Summary of DNA isolation procedures.

Experiment Number	Culture Vessel	Sorbitol Cushion	PEG ppt	Gradient Purification of Virions	Lysis Procedure	Gradient Purification of DNA	Results
1	24 flasks (150 cm ²)	+	-	20-70% Sorbitol CsCl(1.26g/ml)	Sarkosyl/SDS Proteinase K	CsCl/EtBr (1.56g/cm ³)	No DNA detected
2	24 flasks (150 cm ²)	+	-	20-70% Sorbitol Discontinuous CsCl(1.2/1.4g/ml)	Sarkosyl/SDS Proteinase K	CsCl/EtBr (1.56g/cm ³)	No DNA detected
3	24 flasks (150 cm ²)	+	-	Discontinuous Sorbitol (75/48/20%)	SDS Pronase	CsCl (1.72g/cm ³)	Radioactive peak Faint band of DNA on agarose gel
4	24 flasks (150 cm ²)	-	+	Discontinuous Sorbitol (75/48/20%)	SDS Pronase	CsCl (1.72g/cm ³)	Radioactive peak No band of DNA on agarose gel
5	24 flasks (150 cm ²)	-	+	Discontinuous Sorbitol (75/48/20%)	SDS Pronase	CsCl (1.72g/cm ³)	Radioactive peak Faint band of DNA on agarose gel
6 & 7	10 roller bottles (850 cm ²)	-	-	-	Sarkosyl SDS Pronase	-	Total yield 2.5 µg DNA at a concentra- of ~ 12.5 µg/ml

The band was apparent under normal light and was brightly fluorescent under u-v light. There was no fluorescent band of DNA at the appropriate density and no peak of radioactivity. Similar results occurred repeatedly with this procedure.

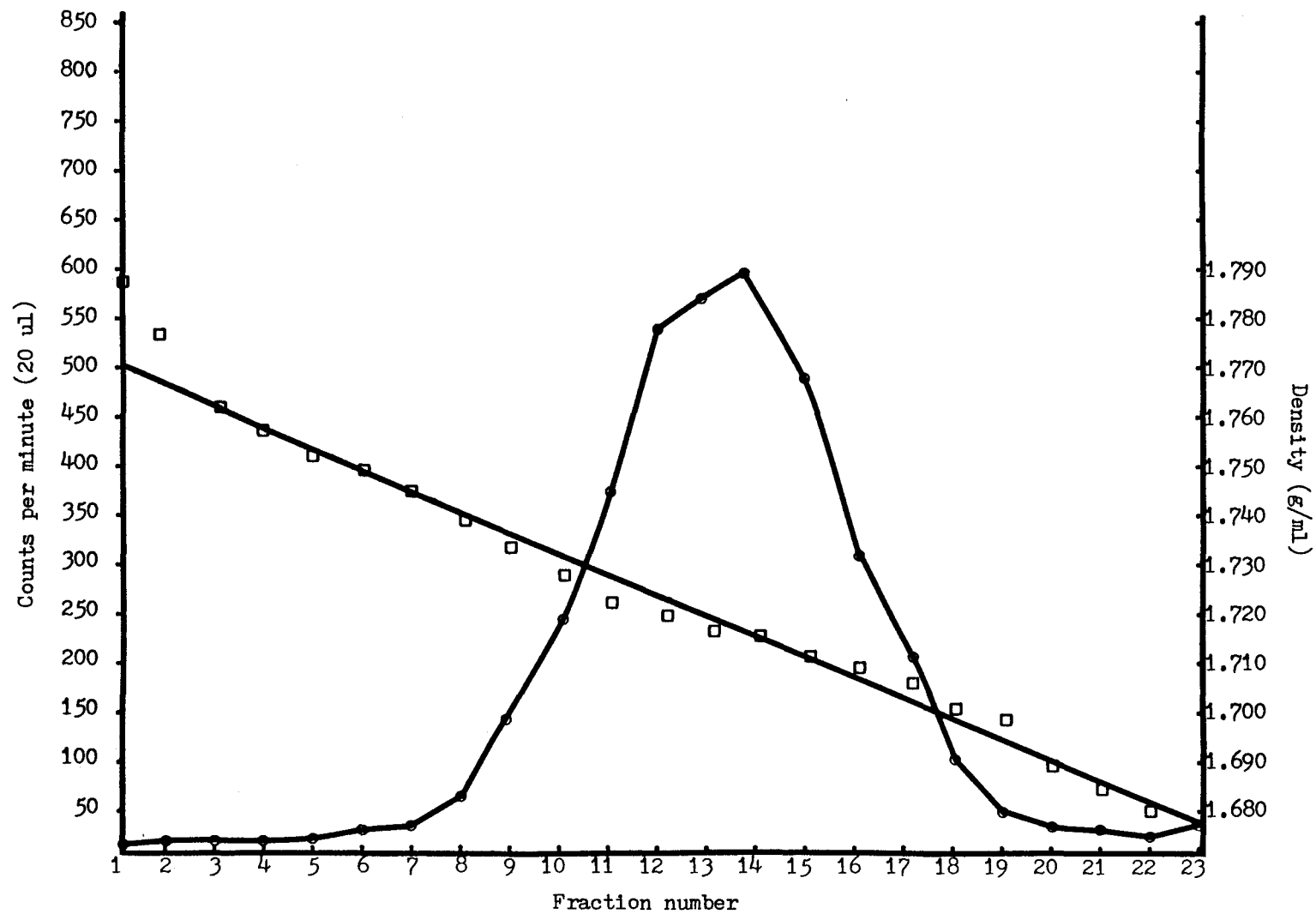
A second lysis procedure was followed in which the virions were treated with SDS, RNase A and pronase (130). The lysate was added to CsCl without ethidium bromide at a density of 1.716 g/cm^3 . This is the density of CMV DNA, which is significantly different from that of cellular DNA (1.69 g/cm^3), thus, there should have been no difficulty in separating viral DNA from any cellular DNA contamination. In fact only a single peak of radioactivity was present in any of the CsCl gradients performed on viral lysates, and this peak was always very close to a density of 1.716 g/ml (Figure 3). In two experiments the material recovered from the CsCl produced a faint band following agarose gel electrophoresis. A rough estimation of the concentration of the DNA based on lambda DNA standards was less than $1 \text{ } \mu\text{g/ml}$. Attempts to concentrate the DNA by ethanol precipitation were, however, unsuccessful. In spite of the fact that there appeared to be a small quantity of DNA extracted in each experiment, there was not enough to perform restriction endonuclease digestion analysis or to clone the fragments.

The method of DNA extraction which was finally successful was a modification of the procedure of LaFemina and Hayward (75). The pelleted virus, which was resuspended in buffer, was first subjected to RNase digestion before lysis of the virions. This differed from the published procedure, which called for the addition of RNase at a later stage in the extraction process. It seemed reasonable to remove contaminating

Figure 3. Fractions from CsCl gradient containing CMV AD169 DNA. The DNA sample was added to CsCl at a density of 1.716 g/cm³ and centrifuged in a Beckman type 50 rotor at 35,000 rpm for 48 h. Fractions (35 drops each) were collected from the bottom, and the radioactivity of 20 μ l aliquots of each fraction was determined.

○ = cpm per fraction for ³H-labelled DNA

□ = density g/ml per fraction



RNA before release of the DNA, since viral lysis should not produce any additional RNA.

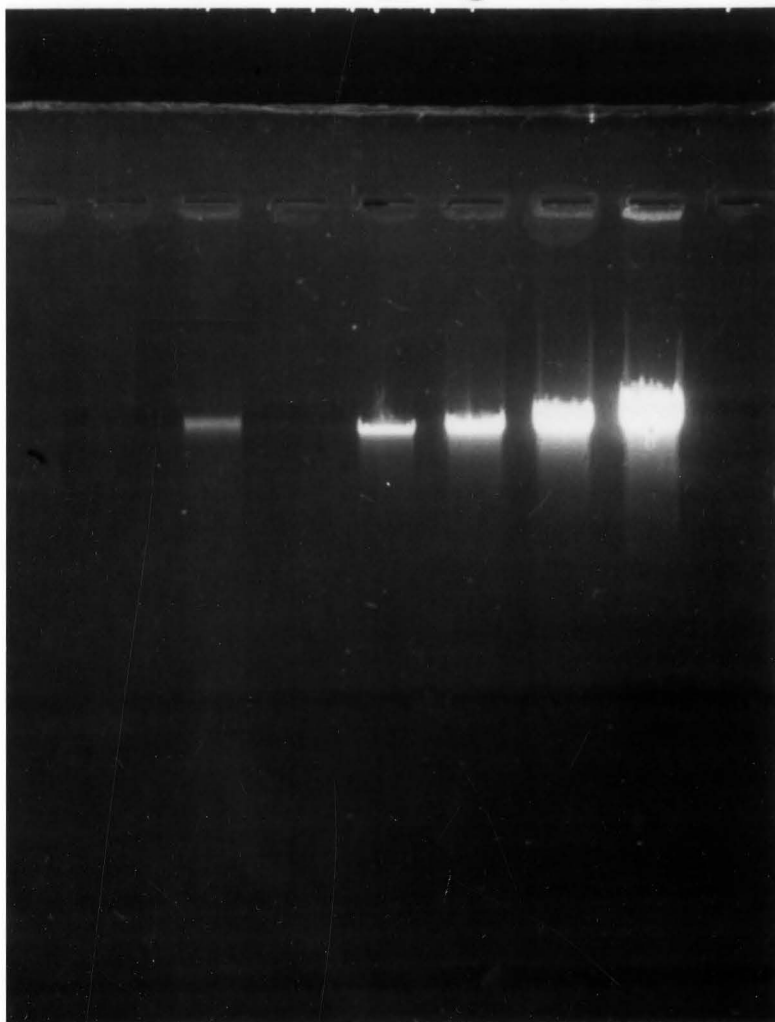
After the RNase treatment the virion suspension was lysed directly with Sarkosyl and SDS, which resulted in almost complete clearing of the fluid. The subsequent addition of pronase removed any residual turbidity. Another modification in the procedure was the incubation time in the presence of pronase. Two hours rather than overnight seemed to be sufficient for the digestion. Following phenol and chloroform extractions the lysate was dialyzed directly without purification in a CsCl gradient. Since no gradient was involved, radioactive labelling of the DNA was not necessary, and there was less chance of losing DNA with additional manipulations. The dialysate was concentrated by ethanol precipitation, and the resulting DNA sample contained approximately 2.5 μg at a concentration of 12.5 $\mu\text{g}/\text{ml}$ (Figure 4).

Isolation of Plasmid pBR322. The plasmid pBR322 was chosen as the cloning vector for CMV DNA fragments. The source of the plasmid was E. coli strain RR1. Following extraction from the bacteria the plasmid DNA was centrifuged in CsCl (1.65 g/ml) with ethidium bromide at 38,000 rpm in a VTi 50 rotor for 38 h. At equilibrium there were two bands about two-thirds of the way down the gradient. The upper portion of the gradient including the upper band containing chromosomal DNA was removed from above. The lower band of plasmid DNA was collected in a total volume of 2.4 ml. The ethidium bromide was extracted with isopropanol saturated with 20X SSC. After overnight dialysis, 5 and 10 μl samples were loaded on an agarose gel along with a known isolate of pBR322 (a gift of Dr. S.K. Farrand). Bands which were assumed to represent linear, open

Figure 4. CMV AD169 DNA after ethanol precipitation. Electrophoresis was performed on a 0.7% agarose gel at a constant current of 40 mA for 3 h. After electrophoresis the gel was stained with ethidium bromide.

Lanes: (1) CMV AD169 DNA
(2) λ DNA 0.125 μ g
(3) λ DNA 0.25 μ g
(4) λ DNA 0.50 μ g
(5) λ DNA 1.0 μ g

1 2 3 4 5



circle and supercoiled plasmid DNA were present in both pBR322 preparations. The corresponding forms of these two plasmid isolates migrated to the same points in the gel indicating that the newly isolated plasmid was probably pBR322.

For further proof of the identity of the plasmid, restriction endonuclease analysis was performed. If all the bands on the gel represented forms of pBR322, then digestion with a restriction enzyme which had only one cleavage site in this plasmid should produce homogeneous linear fragments to form a single band after gel electrophoresis. Double enzyme digests would provide even more information. The plasmid and the known isolate of pBR322 were, therefore, treated with the restriction enzymes HindIII, HinfI or both. HindIII digestion produced a single band of linear DNA, since there is only a single HindIII site in pBR322 (80). This band migrated with the 4.34 kb fragment of a HindIII digest of λ DNA, which would be expected since the size of pBR322 is 4.3 kb. HinfI digestion produced several fragments the largest of which (A fragment) is 1.1 kb in size. This HinfI A fragment has a single HindIII site. As expected double digestion of the plasmid with HinfI and HindIII, produced two smaller bands of the appropriate size from the original A fragment.

The restriction enzyme patterns observed confirmed that the sample plasmid was pBR322. The bands matched those of the standard pBR322 preparation when digested with the same restriction enzymes. In addition the double digest produced the expected change in the HinfI A fragment.

Cloning of CMV AD169 DNA Fragments in pBR322. BamHI was selected

for the cloning procedure, because insertion at the BamHI site in the vector pBR322 consistently inactivates the tetracycline resistance carried by the plasmid (80). The disadvantage of BamHI is that it cleaves the CMV genome into more fragments than other restriction enzymes such as HindIII or XbaI (49,141). E. coli strain SF8 was selected for the subsequent transformation.

The first attempt at cloning produced only twelve transformants of which two were recombinants demonstrating tetracycline sensitivity. One contained a double insert of two small fragments of around 2 kb each, and the second contained a single insert co-migrating with the smaller insert of the other recombinant. The ligation control plates showed no transformants, but the supercoiled pBR322 control DNA produced a large number of transformants. These results suggested that components of the ligation reaction mixtures were inhibiting transformation. During the cloning procedure neither the CMV and vector DNA digests nor the ligation mixture were ethanol precipitated. For the second cloning attempt ethanol precipitation was performed after both of these steps. Of 482 ampicillin resistant colonies 67 were shown to be tetracycline sensitive indicative of recombinant inserts. They have been repeatedly passed on nutrient agar containing 100 µg/ml ampicillin. The sensitivity to tetracycline has been retained indicating that the recombinants are stable.

Analysis of Plasmid Inserts. All of the 67 recombinants were analyzed for plasmid content by the method of Holmes and Quigley (55). On the basis of the migration of the plasmids relative to intact pBR322 the size of the inserts was classified as small, medium or large. Those plasmids which migrated the slowest were selected for restriction diges-

ion with BamHI to determine whether they contained single or multiple inserts. The plasmids pNSL215 and pNSL225 appeared to have the largest single inserts and were chosen for large-scale isolation as potential probes. The reason large fragments were considered desirable was the greater likelihood that they would contain base sequences homologous to all strains of CMV.

A second set of recombinants was analyzed for medium-sized inserts which might contain the U fragment present in the long repeat segments of the CMV genome (45). This fragment lies completely within the long inverted repeats and is, therefore, present in 2 molar quantity in the complete genome digests (Figure 2). Choosing such a fragment as a probe would allow two possible points of homology provided there is not a great deal of variability among CMV strains in this part of the inverted repeat regions. Two recombinants, pNSL45 and pNSL186, were found to have inserts which migrated with the doublet band containing the T and U fragments. These also were subjected to large-scale isolation for further identification.

Identification of Inserted Fragments in Selected Recombinant Plasmids. The first step in the identification of the fragments carried by the four selected recombinants (pNSL45, pNSL186, pNSL215, pNSL225) involved double restriction enzyme digests. The restriction enzyme map of CMV AD169 from Greenaway et al. (49) (Figure 2) was used to choose the appropriate enzymes.

The plasmids pNSL215 and pNSL225 appeared to migrate with the first band of the BamHI digest of CMV DNA. This band contains the co-migrating fragments A and B (15 kb). There was also the possibility

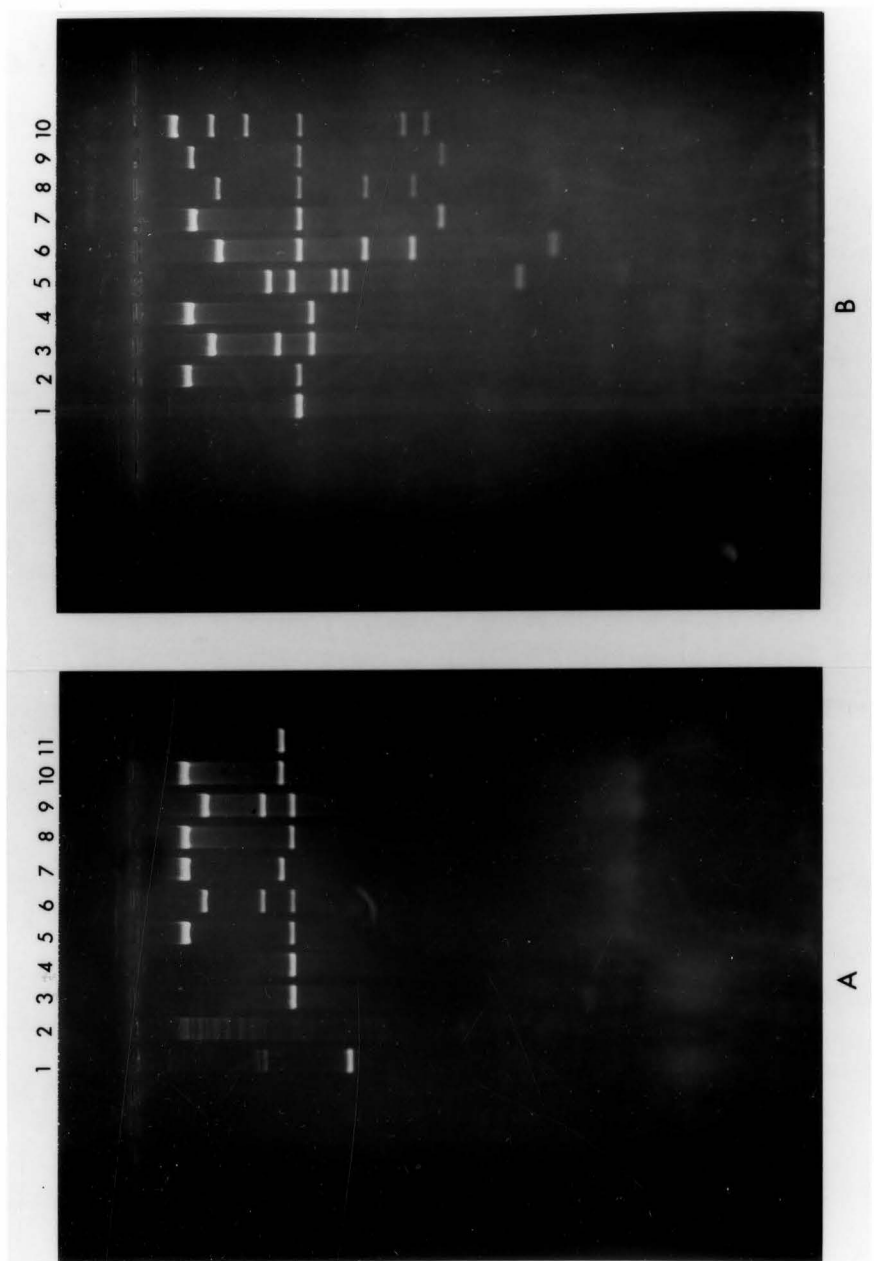
that the closely-migrating fragment C (14 kb) could be the insert. The enzymes EcoRI and HindIII were selected for double digests with BamHI. The A fragment has 3 EcoRI sites and 2 HindIII sites; the B fragment has 1 HindIII site and 1 EcoRI site; the C fragment has 2 HindIII sites and no EcoRI sites. EcoRI and HindIII cut pBR322 alone very close to the same site, thus a double digest of either enzyme with BamHI should yield a larger 4 kb fragment and a smaller 0.3 kb fragment for the pBR322 portion of the recombinant plasmid.

Figure 5A shows the results of these digests. Lane 11 is pBR322 digested with BamHI, which results in a single band of linear DNA. Lanes 3 and 4 are double digests of pBR322 with BamHI/HindIII and BamHI/EcoRI respectively. The major pBR322 fragment (4 kb) is slightly smaller than the single BamHI digested pBR322 as predicted. Lanes 7 and 10 are BamHI digests of each recombinant with the large insert intact. Lanes 6 and 9 are BamHI/EcoRI double digests of each recombinant. In both cases the insert has been cut. Lanes 5 and 8 are the BamHI/HindIII double digests. The inserts do not appear to have been altered by HindIII, because the HindIII recognition site is only about 0.3 kb from the end of the fragment.

To rule out the possibility that the inserts in the recombinant plasmids could be the D fragment, further double digests with BamHI plus PstI, BglIII and XbaI were performed. The digest pattern is shown in Figure 5B. The insert is cut by PstI into three larger fragments plus 2 very small fragments (Lane 5). XbaI cuts the insert only once (Lanes 7 and 9), and BglIII produces 1 larger fragment plus 3 small fragments (Lanes 6 and 8).

Figure 5. Double restriction enzyme digests of plasmids carrying the BamHI B fragment insert.

- A. Lanes: (1) pBR322 uncut; (2) CMV AD169 BamHI; (3) pBR322 BamHI/EcoRI double digest; (4) pBR322 BamHI/HindIII double digest; (5) pNSL225 BamHI/HindIII; (6) pNSL225 BamHI/EcoRI; (7) pNSL225 BamHI; (8) pNSL215 BamHI/HindIII; (9) pNSL215 BamHI/EcoRI; (10) pNSL215 BamHI; (11) pBR322 BamHI.
- B. Lanes: (1) pBR322 BamHI; (2) pNSL215 BamHI; (3) pNSL215 BamHI/EcoRI; (4) pNSL215 BamHI/HindIII; (5) pNSL215 BamHI/PstI; (6) pNSL215 BamHI/BglII; (7) pNSL215 BamHI/XbaI; (8) pNSL225 BamHI/BglII; (9) pNSL225 BamHI/XbaI; (10) λ HindIII.



It can be concluded, therefore, that the double digests of both plasmids are consistent with the pattern which would be predicted for the B fragment. Single HindIII or EcoRI digests were performed on pNSL215 and pNSL225 to determine the orientation of the inserts (Figure 6). Both HindIII digests (Lanes 4 and 5) have two fragments approximately 4.3 kb and 15 kb in size. The EcoRI digests (Lanes 3 and 4) each show fragments of about 9 kb and 10.3 kb. The opposite orientation of the insert in the plasmids would yield HindIII fragments of 0.6 kb and 18.7 kb, and EcoRI fragments of 5.3 kb and 14 kb. The orientation of the inserts is, therefore, the same in these two plasmids. The restriction patterns for both enzymes (HindIII and EcoRI) are consistent with orientation I depicted in Figure 7.

The double digests for the 2 recombinants suspected of carrying the U fragment are shown in Figure 8. The restriction enzymes BamHI plus PstI, EcoRI and HindIII were used to distinguish the U fragment from the QRS (6.5 kb), T (5.6 kb) and V (5.4 kb) bands. The U fragment should have no HindIII or PstI sites and a single EcoRI site (Figure 2). The T fragment has at least 3 PstI sites. Fragments QRS and V all have sites for at least 2 of the 3 enzymes. From Figure 8 it can be seen that both plasmids carry inserts which are not cut by HindIII (Lanes 9 and 13) or PstI (Lanes 7 and 11), but which are cut by EcoRI (Lanes 8 and 12). Again the patterns of the two plasmids appear identical, therefore, both apparently contain the U fragment.

The second approach to the identification of the inserted fragments in the selected recombinants was to produce radiolabelled probes by nick translation of the recombinant plasmids and to hybridize them to

Figure 6. Single restriction enzyme digests of pNSL215 and pNSL225 to determine the orientation of the CMV AD169 BamHI B fragment insert at the BamHI site in pBR322.

Lanes: (1) λ DNA (0.25 μ g) digested with HindIII

(2) pNSL215 EcoRI

(3) pNSL225 EcoRI

(4) pNSL215 HindIII

(5) pNSL225 HindIII

(6) λ DNA (0.75 μ g) HindIII

1 2 3 4 5 6

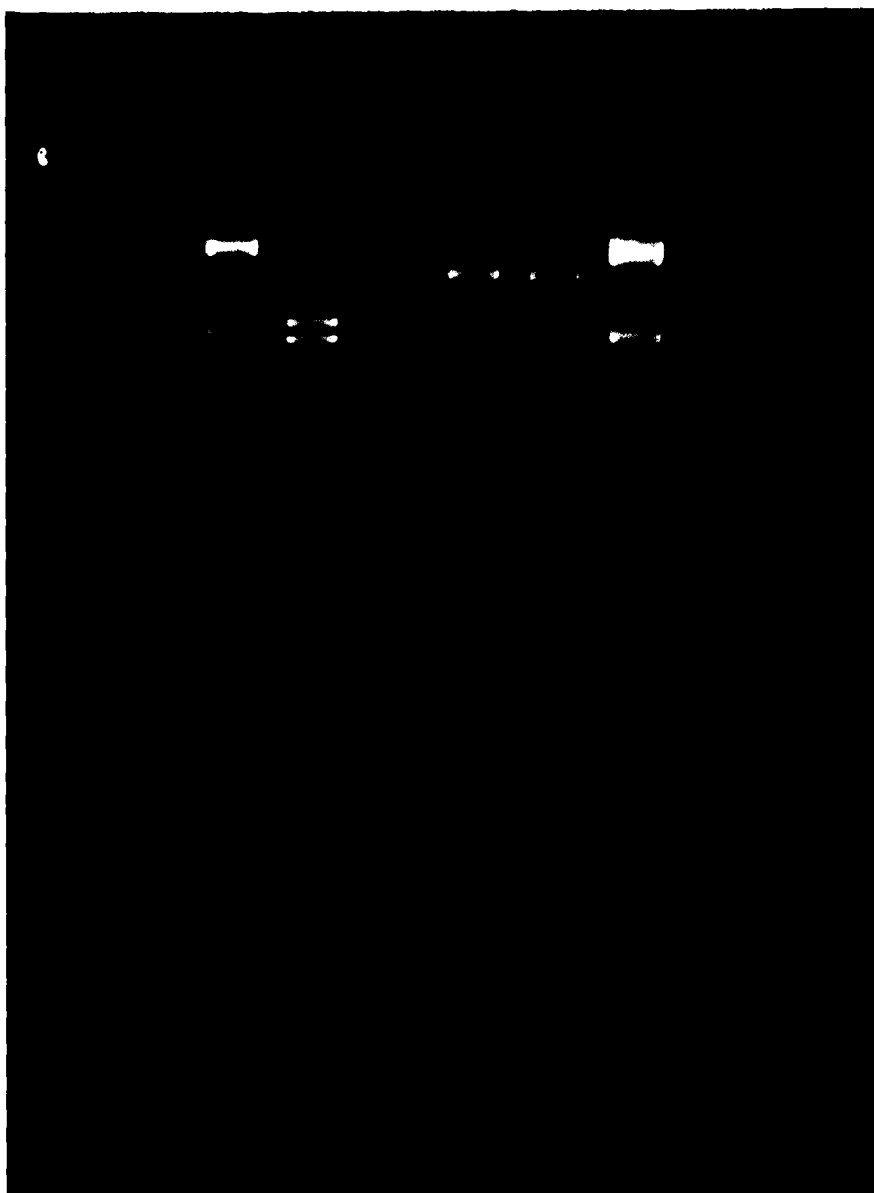
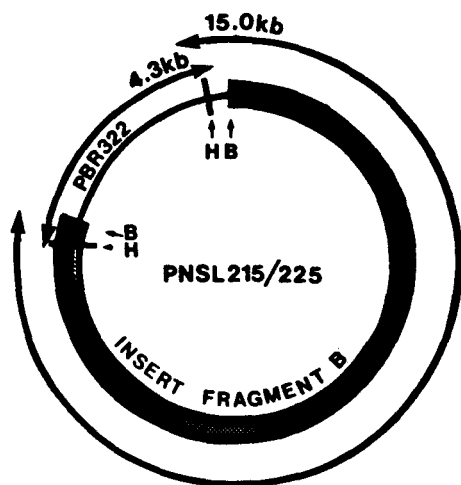
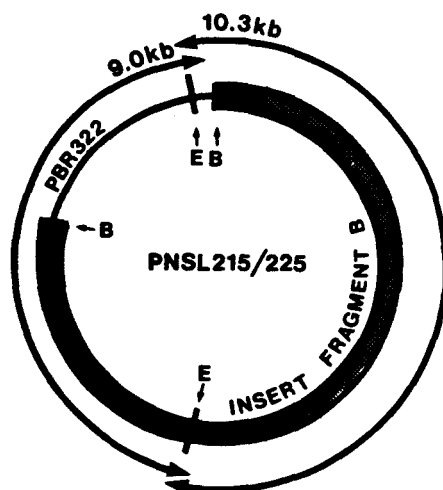


Figure 7. Restriction enzyme maps of pNSL215 or pNSL225 showing fragments expected for each possible orientation. The CMV AD169 BamHI fragment B insert is at the BamHI site in pBR322. The enzyme recognition sites (arrows) are: BamHI (B), HindIII (H) and EcoRI (E). Fragment sizes produced by digestion with either HindIII or EcoRI for each orientation are given.

ORIENTATION I

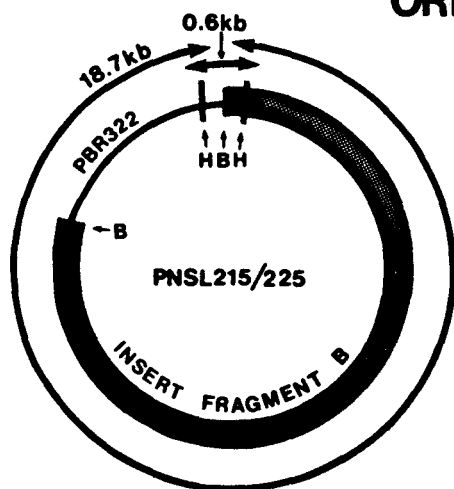


HindIII

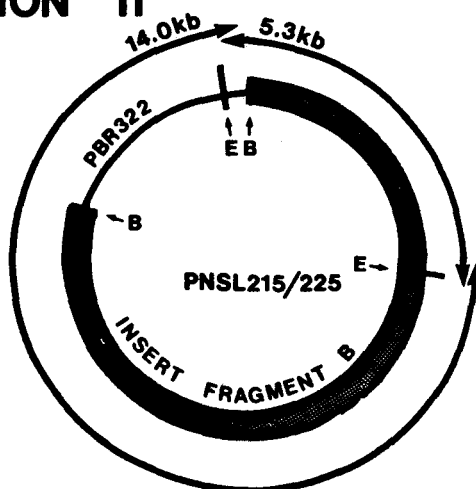


EcoRI

ORIENTATION II



HindIII



EcoRI

Figure 8. Double restriction enzyme digests of plasmids carrying the BamHI U fragment insert.

Lanes: (1) pNSL186 uncut; (2) pNSL45 uncut; (3) pBR322 BamHI/PstI double digest; (4) pBR322 BamHI/EcoRI; (5) pBR322 BamHI/HindIII; (6) pBR322 BamHI; (7) pNSL186 BamHI/PstI; (8) pNSL186 BamHI/EcoRI; (9) pNSL186 BamHI/HindIII; (10) pNSL186 BamHI; (11) pNSL45 BamHI/PstI; (12) pNSL45 BamHI/EcoRI; (13) pNSL45 BamHI/HindIII; (14) pNSL45 BamHI; (15) λ digested with HindIII

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

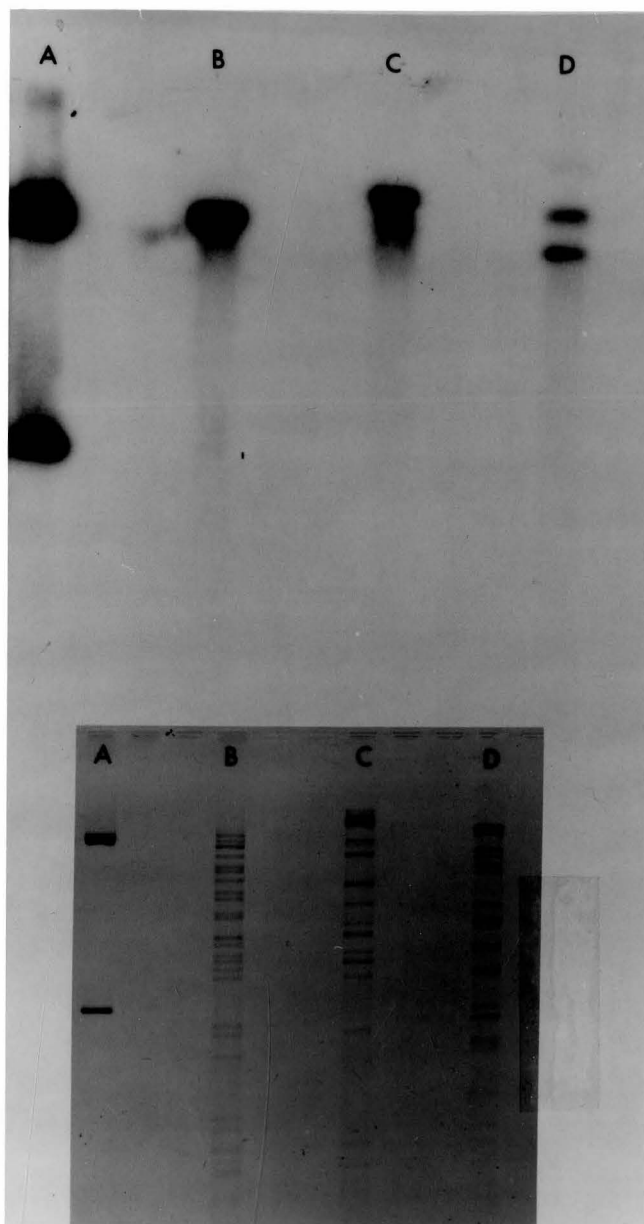


different enzyme digests of total CMV DNA. For the two plasmids pNSL215 and pNSL225 the CMV DNA was digested with EcoRI, HindIII and BamHI. A probe was made with pNSL225. The CMV DNA digests show that the probe binds to a single large BamHI fragment, a large HindIII fragment and 2 large EcoRI fragments (Figure 9). This is consistent with the pattern expected for the B fragment of the BamHI digest. There is a short sequence (≤ 0.3 kb) at the end of the B fragment which would be expected to be homologous to the HindIII L fragment (Figure 2). Hybridization of the probe to this HindIII fragment is not distinguishable in Figure 9, although the existence of a HindIII recognition site in the insert was demonstrated by the HindIII digests of pNSL215 and pNSL225 (Figure 6). The co-migrating BamHI A fragment however, should hybridize to 4 EcoRI fragments and 3 HindIII fragments. The BamHI C fragment should hybridize to only 1 EcoRI fragment and 3 HindIII fragments. The hybridization results (Figure 9) do not support the possible presence of either of these inserts.

The hybridization experiment to identify the U fragment is more difficult to interpret. Since the U fragment is part of the long repeats, it can hybridize to joint fragments comprising the junction of the long and short repeat sequences in other restriction enzyme digests (Figure 1). These joint fragments (L-S) are present in less than 1 molar concentrations, because there are four possible configurations of the CMV genome. The T fragment as well as QRS and V are present only in the unique sequences, and therefore, have better defined hybridization patterns. In Figure 10 it can be seen that the probe which was made from pNSL45 hybridized to several large HindIII fragments some of which appear to be

Figure 9. Southern hybridization of ^{32}P -labelled B fragment probe (pNSL225) with restriction enzyme digests of total CMV AD169 DNA. Autoradiogram shown above with corresponding agarose gel below. Exposure time 19 hours.

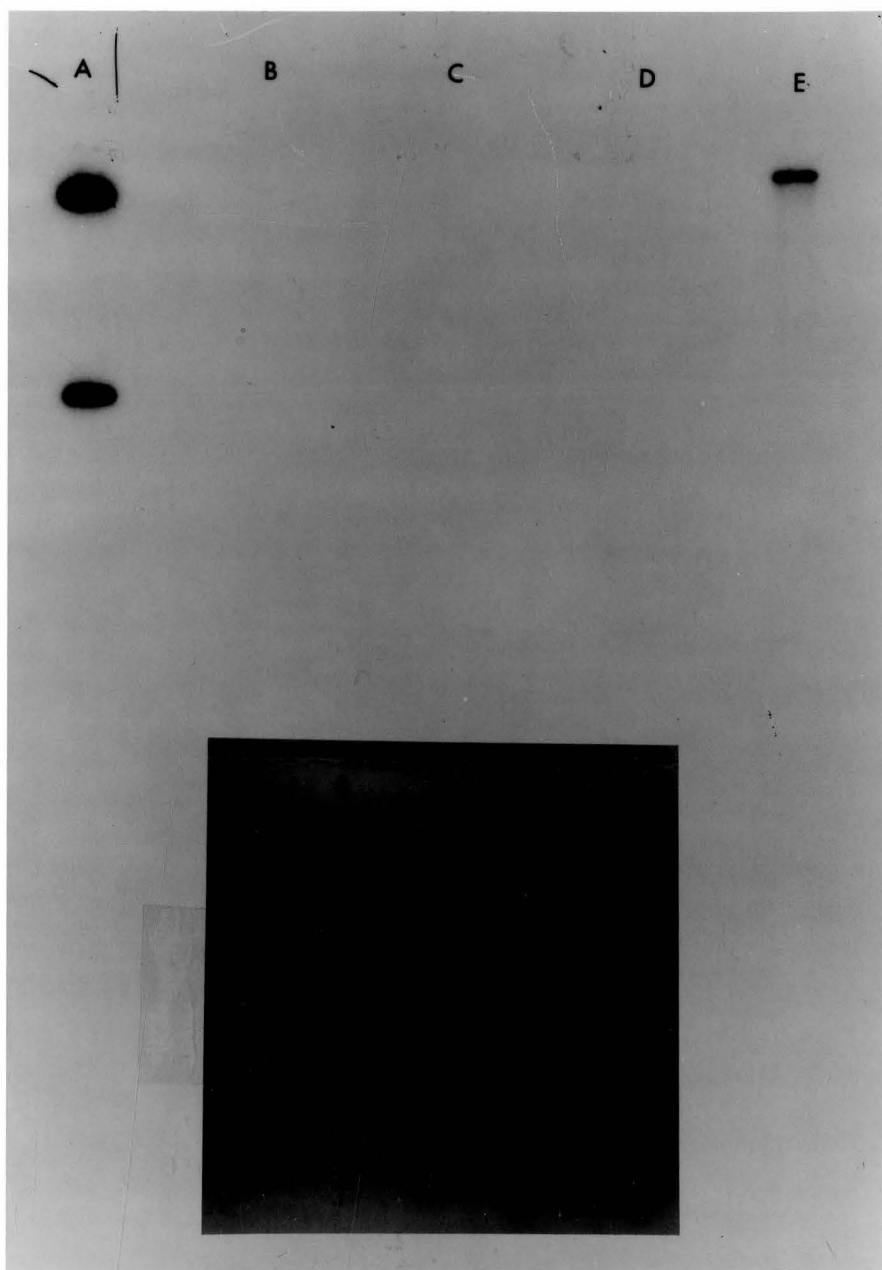
Lanes: (A) pNSL225 BamHI digest; (B) CMV AD169 DNA BamHI; (C) CMV AD169 HindIII; (D) CMV AD169 DNA EcoRI.



placed on the same gels for blotting to nitrocellulose. In Figure 11 it can be seen that the B fragment probe did not hybridize to any fragments of the three viruses, while the appropriate hybridization with the CMV DNA digest clearly occurred. It should be noted that there was less EBV DNA than CMV DNA present on the gel. Similar amounts of CMV AD169 DNA produce strong hybridization with the probe, but there is 100% homology between the CMV DNA and the probe. It could be argued that partial homology with EBV DNA in such low quantity would not be detected. A southern blot using the U fragment probe also showed no hybridization to the DNA digests of these other herpesviruses.

Hybridization of the Probe with Infected Cells. The initial experiment was set up in duplicate using both a nitrocellulose filter and GeneScreen Plus membrane for comparison. MRC-5 cells in 16 x 125 mm tubes were inoculated starting 10 days before the expected harvest date. Two tubes each day were inoculated with a 10^{-5} dilution of stock plaque-purified CMV AD169. On the day of harvest the cells were trypsinized and two ten-fold dilutions were made of the cells from each day of inoculation. The filters were spotted with 5 μ l volumes representing 10^4 , 10^3 , and 10^2 cells from 10 days down to 2 days postinfection. In addition HSV-1, HSV-2, EBV and CMV DNA were spotted. The filters were treated according to the procedure of Brandsma and Miller (7). The nitrocellulose filter, however, was baked only 3 h instead of 18 h, and the GeneScreen Plus membrane was not baked at all. The probe was produced by nick translation of pNSL225. Hybridization of both filters was carried out in heat-seal bags at 65°C for 43 h, but the GeneScreen Plus was constantly agitated in a water bath, while the nitrocellulose was placed

Figure 11. Southern hybridization of ^{32}P -labelled B fragment probe (pNSL225) with HSV-1, HSV-2, EBV and CMV AD169 total DNA BamHI digests. Autoradiogram shown above with corresponding agarose gel below. Exposure time 20 hours. Lanes: (A) pNSL225 BamHI digest; (B) EBV BamHI; (C) HSV-2 BamHI; (D) HSV-1 BamHI; (E) CMV AD169 BamHI.



in an oven and only occasionally flipped and rocked.

In this first attempt to hybridize the CMV probe with infected cells the GeneScreen Plus membrane produced more encouraging results than the nitrocellulose. In both cases there was too much background hybridization, but there were definite spots for 10^4 cells from 10 to 5 days post-infection and for the CMV DNA. The nitrocellulose filter was much more difficult to interpret. The CMV DNA spot was the only definite area of hybridization. It was decided to use the GeneScreen Plus instead of nitrocellulose for future experiments, because it gave better results, was easier to handle and required no baking. The one disadvantage of GeneScreen Plus is the 6-hour minimum prehybridization recommended by the manufacturer.

In the next experiment with infected cells the viral inocula were clinical isolates of CMV. The B fragment (pNSL225) was used as the probe, and the hybridization was performed in the same manner as that outlined for AD169-infected cells. Figure 12 shows that the probe hybridizes to all CMV isolates tested. However, as noted above there is no hybridization to uninfected cells nor to HSV-1 and HSV-2 DNA. From this experiment it can be concluded that the probe can detect more CMV strains than just the stock strain AD169 from which it was made, and there is no cross-reactivity with the 2 strains of HSV which were tested. This suggests that this probe may have both sensitivity and specificity for CMV. The infected cells showed 50-80% CPE depending on the isolate, therefore, no quantitative conclusions can be drawn concerning the degree of sensitivity nor can the possibility of cross-hybridization with other strains of HSV be ruled out.

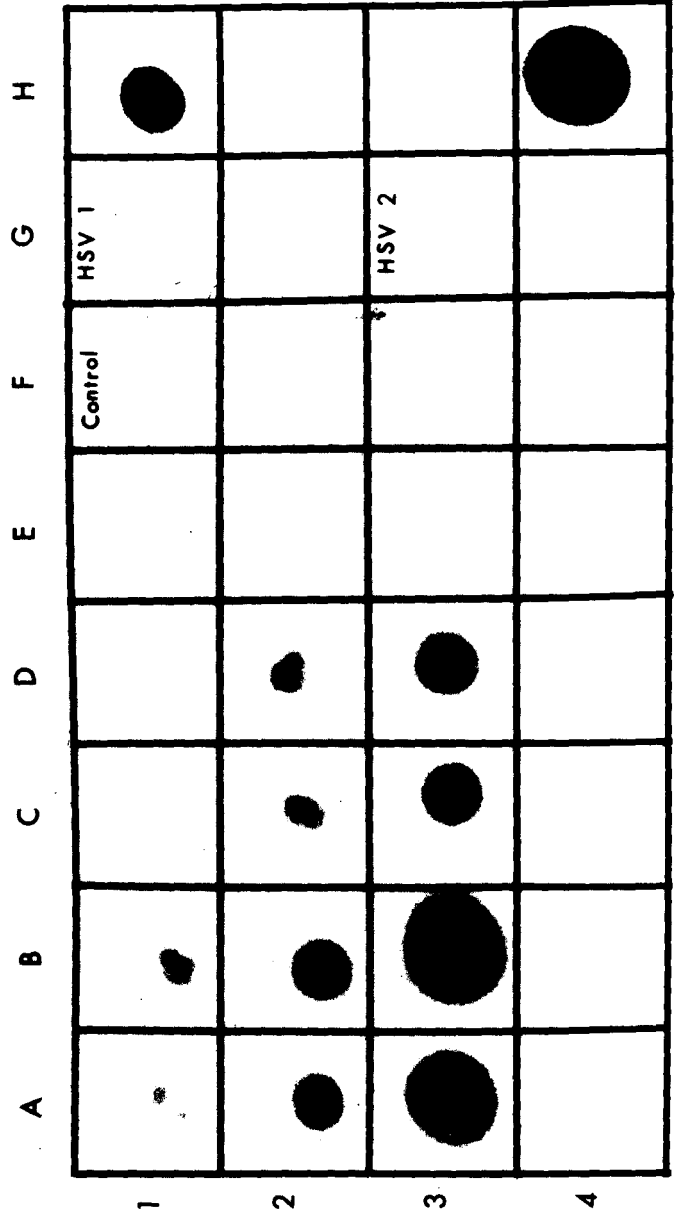
Figure 12. Hybridization of ^{32}P -labelled B fragment probe (pNSL225) with cells infected with CMV AD169 or passaged clinical isolates of CMV. Exposure time 20 hours.

Columns: (A) CMV AD169; (B) LU-1; (C) LU-2; (D) LU-3.

Rows: (1) 1×10^3 infected MRC-5 cells; (2) 1×10^4 infected cells; (3) 1×10^5 infected cells.

Control: 1×10^6 uninfected MRC-5 cells

Squares: (G1) HSV-1 F DNA 0.1 μg
(G3) HSV-2 333 DNA 0.1 μg
(H1) CMV AD169 DNA 0.01 μg
(H4) pNSL225 DNA 0.1 μg



A more quantitative experiment was performed directly on virus particles spotted on the membrane (Figure 13). Dilutions of stock samples of three clinical isolates and AD169 were placed on the membrane and processed according to the method of Brandsma and Miller (7). For one set of dilutions for each virus sample 2.75×10^3 uninfected cells were spotted along with the virus. There appeared to be no significant difference between the virus alone and the virus plus cells for any of the CMV isolates. There was no hybridization with uninfected cells alone. HSV-1 and HSV-2-infected cells were tested along with separate samples of naked HSV-1 and HSV-2 DNA. There was no hybridization with the HSV-infected cells and only slight hybridization with 1 μ g of HSV DNA. There was 50 times as much HSV DNA as control CMV DNA on the membrane, therefore, the background hybridization with the HSV DNA is unlikely to be a problem in other experiments. The number of pfu which the probe detected ranged from 1×10^6 to 2.5×10^3 for AD169, 1.8×10^6 to 4.5×10^4 for LU-1, 4×10^4 to 4×10^2 for LU-2, and 6×10^4 to 6×10^2 for LU-3.

The hybridization of LU-3 with the probe appears as strong as that of AD169 and LU-1. The titer of this isolate, however, is only slightly higher than that of LU-2. A possible explanation of this discrepancy may be that there is a larger number of defective virus particles in the LU-3 preparation. This would yield a greater amount of DNA relative to the number of pfu detected in the plaque assay.

Another experiment was designed to determine how large an inoculum of virus might be required for detection within 72 h of inoculation of cells in culture. Ten-fold dilutions of an AD169 stock sample containing 2×10^7 pfu/ml were used to inoculate MRHF cells in 16 x 125 mm tubes.

Figure 13. Hybridization of ^{32}P -labelled B fragment probe (pNSL225) with cell-free virus or virus plus uninfected cells. Exposure time 23 hours.

Columns: (A) AD169 cell-free virus: 1×10^6 pfu (1); 2.5×10^5 pfu (2); 2.5×10^4 pfu (3); 2.5×10^3 pfu (4); 2.5×10^3 pfu (5).

(B) AD169 cell-free virus plus 1×10^3 uninfected MRC-5 cells (same number pfu as in column A).

(C) LU-1 cell-free virus: 1.8×10^6 pfu (1); 4.5×10^5 pfu (2); 4.5×10^4 pfu (3).

(D) LU-1 cell-free virus plus 1×10^3 uninfected MRC-5 cells (same number pfu as in column C).

(E) LU-2 cell-free virus: 4×10^4 pfu (1); 1×10^4 pfu (2); 1×10^3 pfu (3).

(F) LU-2 cell-free virus plus 1×10^3 uninfected MRC-5 cells (same number pfu as in column E).

(G) LU-3 cell-free virus: 6×10^4 pfu (1); 1.5×10^4 pfu (2); 1.5×10^3 pfu (3).

(H) LU-3 cell-free virus plus 1×10^3 uninfected MRC-5 cells (same number pfu as in column G).

(I) HSV-1-infected MRC-5 cells (1); HSV-1 DNA, 1 μg (2); HSV-2-infected MRC-5 cells (3); HSV-2 DNA, 1 μg (4); pNSL225 DNA 0.2 μg (5).

(Z) Dilutions of CMV AD169 DNA: 10 ng (1); 1 ng (2); 100 pg (3); 10 pg (4). Hybridization on separate membrane under same conditions as samples in columns A-I.

Control (E5): 1×10^3 uninfected MRC-5 cells. CMV AD169 DNA (G5): 20 ng

Figure 13. Hybridization of ^{32}P -labelled B fragment probe (pNSL225) with cell-free virus or virus plus uninfected cells. Exposure time 23 hours.

Columns: (A) AD169 cell-free virus: 1×10^6 pfu (1); 2.5×10^5 pfu (2); 2.5×10^4 pfu (3); 2.5×10^3 pfu (4); 2.5×10^3 pfu (5).

(B) AD169 cell-free virus plus 1×10^3 uninfected MRC-5 cells (same number pfu as in column A).

(C) LU-1 cell-free virus: 1.8×10^6 pfu (1); 4.5×10^5 pfu (2); 4.5×10^4 pfu (3).

(D) LU-1 cell-free virus plus 1×10^3 uninfected MRC-5 cells (same number pfu as in column C).

(E) LU-2 cell-free virus: 4×10^4 pfu (1); 1×10^4 pfu (2); 1×10^3 pfu (3).

(F) LU-2 cell-free virus plus 1×10^3 uninfected MRC-5 cells (same number pfu as in column E).

(G) LU-3 cell-free virus: 6×10^4 pfu (1); 1.5×10^4 pfu (2); 1.5×10^3 pfu (3).

(H) LU-3 cell-free virus plus 1×10^3 uninfected MRC-5 cells (same number pfu as in column G).

(I) HSV-1-infected MRC-5 cells (1); HSV-1 DNA, 1 μg (2); HSV-2-infected MRC-5 cells (3); HSV-2 DNA, 1 μg (4); pNSL225 DNA 0.2 μg (5).

(Z) Dilutions of CMV AD169 DNA: 10 ng (1); 1 ng (2); 100 pg (3); 10 pg (4). Hybridization on separate membrane under same conditions as samples in columns A-I.

Control (E5): 1×10^3 uninfected MRC-5 cells. CMV AD169 DNA (G5): 20 ng

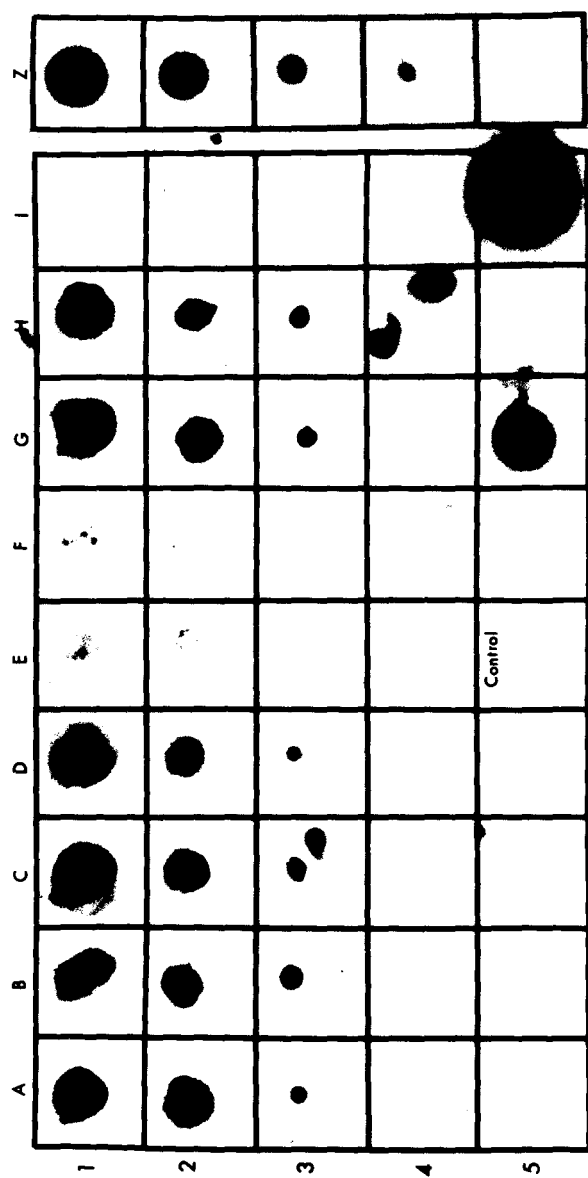


TABLE 4

Correlation of culture results with probe hybridization
to clinical urine specimens

Clinical Specimen Number	Culture	Autoradiogram	Radioactivity of Spotted Membrane (cpm)
1	-	-	61
2	-	-	70
3	-	-	83
4	-	-	58
5	+	+	448
6	-	-	51
7	-	-	75
8	-	-	76
9	-	-	58
10	-	-	95
11	-	-	62
12	-	-	80
13	+	-	70
14	-	-	67
15	-	-	33
16	-	-	Not Done
17	-	-	"
18	-	-	"
19	-	-	"
20	-	-	"
21	-	-	"

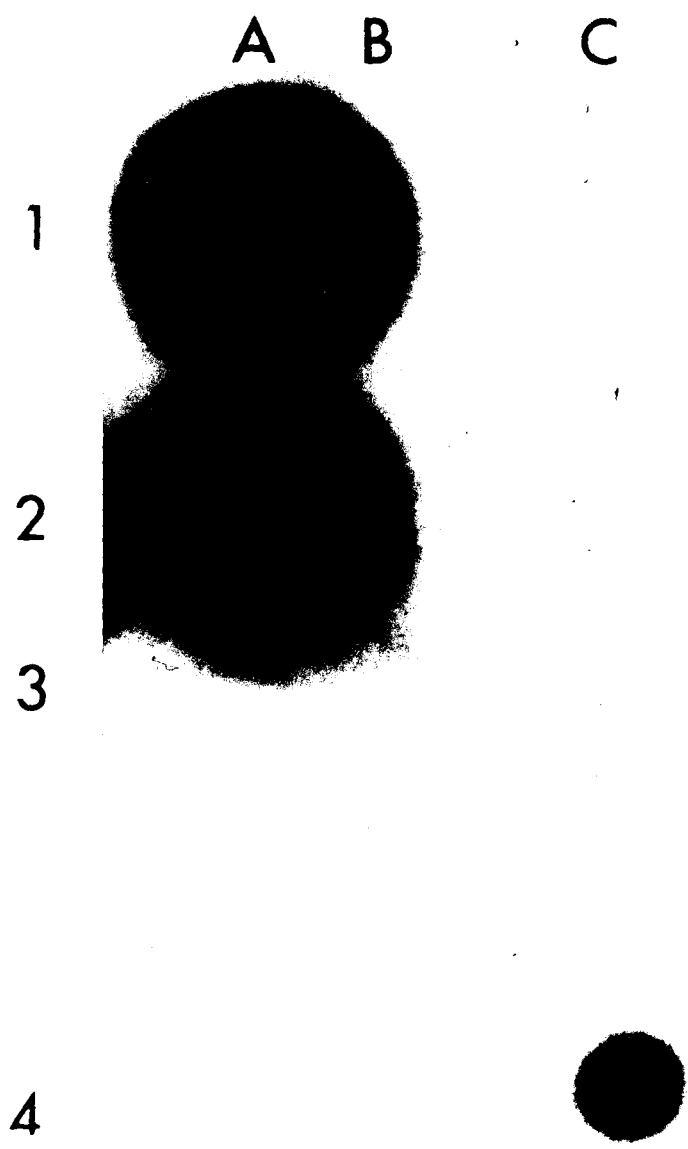
Table 4. (Cont'd)

Clinical Specimen Number	Culture	Autoradiogram	Radioactivity of Spotted Membrane (cpm)
22	-	-	Not Done
23	-	-	"
Background (unspotted membrane sample)			
		-	80
Unlabelled pNSL225 DNA			
(1 μ g in 0.5 ml 1X SCC)		+	133,280
Unlabelled pNSL225 DNA			
(1 μ g spotted directly)		+	29,051

passage of the original inoculated monolayer, was also negative with the probe. The second culture-positive specimen was from a patient with suspected AIDS. The original culture was positive within 72 h and both the autoradiogram and scintillation counting from the prepared urine specimen showed strong binding of the probe (Figure 14). The autoradiogram in Figure 14 also contains two of the culture-negative clinical specimens which fail to bind the probe. There are two faint areas of hybridization which correspond to cells from monolayers infected with HSV-1 and HSV-2. This was the only experiment in which there was background hybridization with these two viruses. The HSV samples contained cells whereas the clinical specimens were cell-free. It may be that the processing of the membrane during this experiment was inadequate to remove cellular material which might nonspecifically bind the probe. In addition, a different cell type (MRHF) was inoculated with the viruses. Previously MRC-5 cells had been used. The possibility of probe binding to MRHF cells should be investigated for any future experiments which require virus-infected cells.

The procedure for processing the clinical specimens was a modification of that of Chou and Merigan (13). The salt concentration of the NaOH/NaCl solution used to disrupt the pelleted virions was lowered from 2M to 0.3M such that the final concentration of the specimen to be filtered would be equal to that of 1X SSC (0.15 M NaCl). This change was instituted on the advice of the GeneScreen Plus manufacturer (New England Nuclear) to achieve optimal binding of DNA to the membrane. To check for adequate binding of DNA to the membrane 1 μ g of unlabelled probe DNA was added to 0.5 ml 1X SSC and filtered with the Minifold apparatus. Another

Figure 14. Direct hybridization of B fragment probe to patient urine specimens. The large spots in column A represent 1 μ g of unlabelled pNSL225 DNA either spotted directly (row 2) or placed in 0.5 ml 1X SSC and filtered onto the membrane (row 1). Faint spots in row 3 of columns B and C are HSV-1 and HSV-2-infected MRHF cells. The large spot in column C row 4 is the sample from the suspected AIDS patient. Two other culture-negative clinical samples were spotted in row 4 columns A and B but did not bind the probe. Exposure time 23 hours.



1 μ g was spotted directly on the membrane. Both DNA samples hybridized to the probe equally (Figure 14).

Biotin-Labelled Probe. The biotin-labelled probe was first hybridized to a duplicate membrane corresponding to Figure 12. For both stock AD169 and the three clinical isolates, 10^5 infected cells could be visualized. For the AD169 and LU-1 samples, 10^4 infected cells also produced an easily visible color reaction. The corresponding dilutions of LU-2 and LU-3 were very much fainter. All other dilutions were negative as was the control containing 10^6 uninfected cells. The CMV and pNSL225 DNA controls were positive, and HSV-1 and HSV-2 DNA spots were negative. These results suggested a 10-100 fold lower degree of sensitivity for the biotin probe over the corresponding radiolabelled probe.

The next membrane to which the biotin probe was hybridized was a duplicate of that in Figure 13. The probe failed to detect any of the virus or virus plus cells samples. Probe and CMV DNA were of the same color intensity as in the previous experiment. There was no hybridization to HSV-1 or HSV-2 DNA nor to the control cells. The total lack of color reaction for any of the virus samples was unexpected, since all samples were readily detected with the radiolabelled probe. Both membranes were inoculated at the same time and prehybridized using the Gene-Screen protocol. The same biotin probe sample from the previous experiment was reused for this membrane, which is in agreement with the manufacturer's recommendations. The positive controls seemed to be valid. From this experiment it would appear that the biotin probe is even more than 100-fold less sensitive than the radiolabelled probe. It should be noted, however, that the amount of CMV DNA present in heavily infected

cells far exceeds the amount of DNA which would be released from cell-free virus stocks. Thus the amount of DNA on the membrane in the previous experiment is much greater than that in this experiment. The difficulty in interpretation arises from the direct comparison of the biotin and radiolabelled probes in this experiment.

The biotin probe was next hybridized to the same dilutions of naked CMV DNA seen in Figure 13. A visible color reaction was present for the 100 pg but not for the 10 pg dilution. This represents more than a 10-fold difference in sensitivity, since it would appear from the autoradiogram that less than 10 pg of DNA might be detected, and the 100 pg spot on the biotin probe membrane was very faint.

The biotin probe was hybridized to the first 11 of the 23 clinical urine specimens. Variable color reactions occurred in the inoculated areas with no correlation to the culture or autoradiographic results. The specimen from the suspected AIDS patient showed the same amount of color development as several culture-negative specimens. Some of the other culture-negative specimens had background color produced by substances present in the urine sediment.

Only 20% of each prepared clinical urine specimen was actually spotted on a membrane for hybridization to the biotin probes. Sixty percent of the specimen was filtered with the Minifold onto a membrane for hybridization to the ^{32}P -labelled probe. In Figure 14 it can be seen that the culture-positive specimen from the suspected AIDS patient was positive by autoradiography, and that other culture negative specimens were negative by this technique as well. Comparison of the hybridization of the biotin and ^{32}P -labelled probes

with culture results of these clinical specimens suggests that the biotin-labelled probe binds non-specifically, while the ^{32}P -labelled probe hybridization correlates with the culture results.

Another experiment was performed to try to quantitate the biotin probe sensitivity with stock virus dilutions. Samples of AD169, LU-1, LU-2 and LU-3 stock cell-free virus were placed in the Minifold filtration apparatus and applied to a GeneScreen Plus membrane. The corresponding pfu for each dilution are listed in Table 5. It would appear that the probe can detect 1×10^5 pfu. This can be compared to Figure 13 where the radiolabelled probe detected 1×10^3 pfu of LU-2. To make sure that the membrane would bind DNA in solution during the filtration process, 1 μl of pNSL225 DNA was diluted with 0.5 ml 1X SSC and placed in a Minifold well. Another 1 μl of the pNSL225 DNA was spotted directly onto the membrane. Both samples appeared equally clearly on the membrane after hybridization. This experiment again suggests at least a 100-fold difference in sensitivity between the biotin and ^{32}P -labelled probes.

No firm conclusion concerning the relative sensitivity of the biotin probe should be made, however, since there were some technical variations and difficulties in following the manufacturer's protocol. The protocol was revised by Enzo-Biochem for the amount of probe DNA required for hybridization. There were also changes made by the manufacturer in the hybridization method, which may account for some of the variability of the results. For example the final membrane washing with 2X SSC after color development was added to the original protocol. This step reduces the background color. In addition two different

TABLE 5

Quantitation of biotin probe sensitivity

	<u>Virus pfu</u>	<u>Color Development</u>
AD169	1×10^6	++
	1×10^5	<u>+</u>
	1×10^4	-
	1×10^3	-
LU-1	1.8×10^6	++
	1.8×10^5	<u>+</u>
	1.8×10^4	-
	1.8×10^3	-
LU-2	1.5×10^5	<u>+</u>
	1.5×10^4	-
	1.5×10^3	-
	1.5×10^2	-
LU-3	1×10^5	<u>+</u>
	1×10^4	-
	1×10^3	-
	1×10^2	-
Control	pNSL225 DNA (1 μ g)	++++

methods were used for retrieving the probe following nick translation. The original probe samples were placed on a 3.5 ml Sephadex G-50 column and fractions were collected. Since ^3H -labelled ATP was added to the nick translation reaction in addition to cold ATP, the incorporated label could be used to select the fractions containing the probe.

Another method was the use of a 1 ml Sephadex G-50 spin column (Enzo-Biochem protocol). Theoretically the 50 μl nick translation volume should be recovered in approximately equal volume after centrifugation in an Eppendorf tube at the tip of the Sephadex column. The actual volume which was collected in 4 different nick translation reactions varied from 200-500 μl . To check that the ^3H -labelled probe was separated from unincorporated nucleotides, one of the probe samples was placed on a 3.5 ml column and fractions were collected as before. There was only one peak of radioactivity, which occurred right behind the void volume; therefore, the probe had been separated but was present in too large a volume. The samples were each ethanol precipitated; recounting indicated that the probe was concentrated. It may be, however, that more probe was lost during the purification steps of the spin column technique than during purification on the 3.5 ml Sephadex column. A loss of biotinylated probe would have reduced the apparent sensitivity of the hybridizations in the last experiment described above.

DISCUSSION

Tissue culture has been the standard method for virus isolation and identification in the laboratory. The method is both specific and sensitive in that a single plaque-forming unit of virus can be amplified from the original inoculum to produce visible CPE. However, replication of many viruses is very slow in tissue culture; the method is expensive, and not all viruses can be easily propagated in cell types commonly used for virus screening.

Recently the need for more rapid diagnostic tests for viral infections has increased with the introduction of antiviral chemotherapy. Many of the currently approved drugs have very narrow spectra of antiviral activity (53), which makes identification of the etiologic agent important for treatment of each individual viral disease. In addition monitoring of the progress of treatment requires rapid laboratory results (13,81,105,118). Rapid identification of viral disease can also curtail the need for extensive diagnostic testing and aid in the epidemiologic control of virus dissemination (105).

The use of nucleic acid probes shows promise as a technique for rapid viral detection and identification. Richman et al. (105) were able to detect 1 pg of purified HSV DNA or 10^4 pfu of cell-free virus with several HSV probes. Herpes simplex type specificity was achieved with probes carrying fragments from the junction region of the genome (105) or by increasing the stringency of the hybridization conditions (122). Similar probes developed for the detection of EBV (3,7) were

able to detect 2-5 pg of DNA. Hybridization methods for identification of adenovirus (134) and human papilloma virus (16) have been recently reported. Probes for RNA viruses have also been developed. Type-specific probes for rotaviruses were shown to hybridize to 8 pg of homologous double-stranded RNA (33). A copy DNA probe containing the replicase gene of coxsackievirus B3 could detect other enteroviruses in tissue culture inoculated with stool specimens (63). Hybridization in culture occurred with coxsackieviruses A and B, echovirus, and poliovirus. The sensitivity, however, was not sufficient to detect these viruses directly in the original specimen.

The slow growth of CMV in routine laboratory tissue culture has prompted a search for faster methods for detecting this virus, and DNA-DNA hybridization has been one line of investigation (13,81,118). The goal of this project has been the development of a DNA probe for rapid detection and identification of CMV. Several steps were required in the development process: 1) isolation of CMV DNA from strain AD169; 2) cloning of BamHI restriction endonuclease fragments of the viral DNA; 3) selection and identification of an appropriate cloned fragment for a probe, and 4) determination of the sensitivity and specificity of the radiolabelled and biotin-labelled probe for CMV.

Cytomegalovirus AD169 is a well-characterized laboratory strain for which there is a restriction map available (49). This strain was used as a source of viral DNA, because the virions are released extracellularly. Typical wild-type CMV strains remain highly cell-associated. The extracellular virions provide a source of viral DNA which is free of cellular DNA. Cellular debris can be removed by low-speed centrifuga-

tion before the virus particles are pelleted from the supernatant fluid. The method of DNA isolation which finally allowed the production of enough DNA for cloning did not require further purification of the pelleted virus (76). The virions were lysed directly after resuspension in a small amount of buffer. The lysate was extracted twice with organic solvents and then dialyzed. No gradient was required at any point during the procedure, and DNA suitable for restriction enzyme analysis was obtained. The BamHI restriction endonuclease digestion pattern (Figure 9) of the isolated CMV DNA matches that published by Huang et al. (60) for strain AD169. Furthermore the lack of a fluorescent background suggests that the CMV DNA is chemically pure and not contaminated with other DNA species.

The plasmid vector pBR322 was chosen for its ready availability and well-characterized restriction map (18,82). BamHI was chosen as the enzyme to produce DNA fragments for cloning, because inactivation of tetracycline resistance by insertion at the BamHI site in pBR322 provides a consistent screening marker for recombinant plasmids. HindIII has been used by others (75) to produce cloned CMV fragments. The advantage of this enzyme is that it cuts the DNA into fewer fragments than does BamHI, and the largest fragments of the HindIII digest are considerably larger than the largest BamHI fragments. The disadvantage of HindIII is that insertion at the HindIII site in pBR322 does not consistently inactivate tetracycline resistance. Cytomegalovirus AD169 DNA fragments have been cloned into the EcoRI site in pACYC184 (129). EcoRI also has fewer restriction sites in the CMV genome, but there is no selection marker for insertion at the EcoRI site in pBR322. The

largest EcoRI fragments are not much larger than the largest BamHI fragments; therefore, there seemed to be no advantage to using the EcoRI/pACYC184 system. Insertion at the PstI site inactivates the ampicillin resistance marker of pBR322, but two of the largest restriction fragments produced from CMV AD169 DNA are found in the junction region of the genome. These fragments are present in only 0.25 M quantity in the total CMV DNA digest, which means that only one-fourth of the viral genomes contain the intact fragment sequence. This enzyme also produces significantly smaller fragments than does BamHI. The enzyme which produces the largest and fewest restriction fragments in CMV AD169 DNA is XbaI, but there is no restriction site for this enzyme in pBR322. Thus, considering all of the commonly used enzymes and vectors, BamHI with pBR322 seemed to be the most convenient combination to use for cloning.

A cloned fragment to be used as a probe should contain DNA sequences which are common to all HCMV strains and which are present in all configurations of the genome. There were, therefore, two types of recombinant plasmids of interest. First, a plasmid with a large single insert preferably the A or B fragment of the BamHI digest was sought for its long unique sequence. Second, the U fragment was considered desirable for its 2 molar concentration in the total CMV AD169 genome. Two plasmids (pNSL215 and pNSL225) were selected for further study, because they had large single inserts. Both plasmids turned out to carry the B fragment in the same orientation (Figure 7). This identification was established by both restriction endonuclease digestion patterns of each plasmid insert and hybridization of the radiolabelled recombinant

plasmid with restriction endonuclease digests of total CMV AD169 DNA (Figures 5,6 and 9).

Two other cloned plasmids (pNSL45 and pNSL186) were chosen for analysis, because their single inserts migrated with the band of the CMV BamHI digest which contained the U fragment. Both plasmid inserts were subsequently confirmed to be the U fragment. Again restriction endonuclease digestion patterns of the inserts and hybridization of the ³²P-labelled plasmid with total CMV digests were the methods used to establish the identity of the cloned fragments (Figures 8 and 10).

Based on recent reports of variability among CMV strains in the repeat regions of the genome (75,143) and sequences in the repeats, which were at least partially homologous to cellular DNA sequences (85), it was decided that the U fragment in the IR_L might not be desirable for a probe. The B fragment is found in the U_L segment of the genome in a region that has not yet been reported to be either variable among CMV strains or homologous to cellular DNA (15,86,94,119). This fragment was, therefore, chosen for all subsequent experiments.

It was obvious from the fragment identification experiments, that the probe containing the B fragment hybridized to CMV strain AD169 DNA (Figure 9). This probe was then tested for its ability to hybridize with DNA from laboratory strains of HSV-1 (strain F), HSV-2 (strain 333) and EBV (strain B95-8). The Southern blots showed no cross-reactivity of the probe with any segment of these DNA digests (Figure 11), although, as previously mentioned, there was only about half as much EBV DNA as CMV DNA present on the filter. The only other human herpesvirus which would be of clinical significance is VZV, but unfortunately no

DNA from that virus was available for testing. We can conclude from these experiments that the CMV B fragment probe shows no detectable hybridization with DNA from these strains of HSV-1, HSV-2 and EBV. It still remains possible that the DNA from other isolates of these viruses or from VZV could cross-react with the probe. However, other reports (59,94) suggest it is unlikely that under the conditions of hybridization used in these experiments, there would be sufficient homology between the genomes of these viruses and CMV to alter the specificity of the probe. In converse experiments, Richman et al. (105) demonstrated that HSV probes did not hybridize with CMV- or VZV-infected cells.

Since the probe was a construct containing a unique sequence DNA fragment of CMV strain AD169, it was necessary to demonstrate that it could hybridize with other strains of CMV. The three clinical isolates which were initially tested were obtained from specimens submitted to the clinical virology laboratory for culture. Repeated passage of infected monolayers eventually led to the production of 100% CPE with release of infectious virus into the culture supernatant fluid. The probe could then be used to detect both cell-free virus and virus-infected cells (Figures 12 and 13). The probe hybridized very strongly to these isolates under either cell-free or infected-cell conditions. These results do not prove conclusively that the probe can detect all human strains of CMV, but there appears to be sufficient homology reported among HCMV strains (58,142) to suggest that the probe should be able to detect all clinical isolates.

Quantitative experiments were designed to determine the amount of CMV DNA and the number of plaque-forming units of intact virus re-

quired to produce a visible reaction by autoradiography. The 10 pg sample of CMV AD169 DNA could easily be seen on an autoradiogram after 20 hours of exposure (Figure 13). This represents about 4×10^4 genomes of CMV. The degree of sensitivity in the picogram range is in agreement with that reported by Chou and Merigan (13). No purified DNA from strains of CMV other than AD169 was available for testing; therefore, the sensitivity of the probe could differ among CMV strains, if there were any variability of the sequences homologous to the AD169 BamHI fragment. The evidence from the experiment using dilutions of cell-free virus from the clinical strains LU-1, LU-2 and LU-3 (Figure 13), however, suggests that the B fragment probe is probably nearly as sensitive for strains other than AD169. The intensity of hybridization of the probe with each of these clinical strains correlates with the number of pfu spotted on the membrane. For example the three dilutions of strain LU-1 represent 1.8×10^6 , 4.5×10^5 and 4.5×10^4 pfu respectively, and the intensity of the spots on the autoradiogram decreases with each dilution. The lowest number of pfu detected with the probe is 1×10^3 pfu represented by the highest dilution of strain LU-2. This degree of sensitivity is similar to that reported by others (13).

The amount of CMV DNA which is actually present for each dilution in Figure 13 is difficult to determine, since the ratio of pfu to viral particles is very low for CMV and may well vary from one preparation of virus to another. The many noninfectious particles may contribute DNA which will increase the intensity of probe hybridization. A correlation between pfu and probe sensitivity may, therefore, be misleading without electron microscopy to show the number of viral particles con-

taining DNA. In addition there may be many virions containing defective DNA, which might include a deletion of some or all of the B fragment sequences. The evidence presented here, however, generally indicates that the radiolabelled probe is likely to detect at least the majority of HCMV strains with a reasonably high degree of sensitivity.

In comparison with the ^{32}P -labelled probe the sensitivity of the biotinylated probe was found to be at least 100-fold lower when the nick translation was performed according to the protocol published by Enzo-Biochem. Dilutions of CMV AD169 DNA could barely be visualized at the 100 pg level whereas the radiolabelled probe was easily able to produce a dark spot on the autoradiogram with 10 pg of DNA. Equivalent membranes carrying dilutions of stock virus samples (Figure 13 and Table 5) also showed the much lower sensitivity of the biotin probe as compared to the ^{32}P -labelled probe.

The next step was to hybridize the radiolabelled probe to actual clinical specimens to see if the probe could directly detect CMV before inoculation of tissue culture. Samples from urines sent to the clinical laboratory for CMV culture were processed for autoradiography. A multiple filtration apparatus (Schleicher and Schuell Minifold) was used so that all of the pelleted sample could be spotted on the membrane. The results of the hybridizations with the clinical specimens show that virus present in urine can be pelleted and detected directly with the radiolabelled probe (Figure 14), but quantitative levels of detection have not been determined. One specimen, which was positive by both culture and autoradiography was from a suspected AIDS patient, who appeared to be excreting a high titer of virus. This was indicated by the fact

that CPE was present within 72 hours after culture inoculation. The one specimen which was culture-positive but negative by autoradiography and scintillation counting had a very low viral titer. The initial culture was negative and a blind passage became positive with very few infectious foci after several weeks of incubation. All other urine specimens from this patient have been culture-negative for CMV. The remaining clinical specimens (21 out of 23) tested were culture-negative and showed no hybridization with the probe.

With only two known culture-positive clinical specimens available the probe has not been adequately tested for direct detection of CMV in clinical samples. The data so far only indicate that the probe is capable of detecting clinical strains of CMV when the virus is present in quantities sufficient to produce CPE in the initial culture.

Other investigators have successfully used different CMV probes for direct detection of the virus in clinical specimens. Chou and Merigan (13) used the cloned CMV AD169 EcoRI O fragment as a ³²P-labelled probe for hybridization to urine specimens and were able to detect virus at a titer of 10^3 or higher in the original sample. They reported no false-positive results. Spector et al. (118) hybridized ³²P-labelled probes carrying the CMV AD169 EcoRI B and D fragments to buffy coat specimens from bone-marrow transplant patients. They reported that the DNA-hybridization technique was more sensitive than tissue culture for detecting CMV infection in these patients. Marlowe et al. (81) could demonstrate the presence of CMV in 19 of 20 culture-positive urines as well as three lung biopsy specimens. Their ³²P-labelled probe contained cloned fragments of CMV Towne DNA.

The use of ^{32}P for detection of probe hybridization can provide both sensitivity and rapid results. Low levels of hybridization can be visualized by holding autoradiograms for longer periods of time. Scintillation spectrometry can be used in place of autoradiography to decrease the time required for testing (13). The disadvantages associated with the use of ^{32}P are the short half-life (14 days) and the high radiation energy hazard of the isotope.

By contrast the main advantages of the biotinylated probe are the lack of radioactivity and the stability of the probe. According to the manufacturer the biotin label is stable for at least a year. The Enzo-Biochem nick translation procedure, however, also calls for the incorporation of a small amount of ^3H -labelled ATP as well as cold ATP. The radioactivity is used as a tracer to facilitate the recovery of the probe from the unincorporated nucleotides on a Sephadex G-50 column. Presumably the radiolabel can be eliminated, if either a spin column or ethanol precipitation method is used for probe recovery. The Enzo-Biochem protocol unfortunately does not address the problem of the undesirability of the tritium label.

In addition to lower sensitivity non-specific binding in the presence of cells or clinical material made the biotinylated probe unsuitable for direct detection of the virus. Technical difficulties, however, may be responsible for the unsatisfactory results with this reagent. The conclusion drawn from the experiments for this project is that the ^{32}P -labelled probe is at the moment much better than the biotinylated probe for detection of CMV.

There are recent reports, however, which suggest that modifica-

tions in the biotin-labelling procedure can increase the sensitivity of these probes. Leary et al. (78) used alkaline phosphatase instead of hrp for visualization of probe hybridization. In an homologous plasmid reaction 3.1 pg of DNA could be detected. Richman et al. (105) used this modification and were able to detect 1 pg of HSV DNA, a level of sensitivity which is equivalent to that of ^{32}P -labelled probes. The biotinylated probe for EBV constructed by Sixbey et al. (112) was detected with fluorescein-isothiocyanate-labelled avidin and was found to be more sensitive than a comparable probe labelled with ^{32}P .

Directions for Future Research. The evidence so far suggests that the CMV BamHI B fragment probe will detect the majority of HCMV strains, but further testing will be required to demonstrate that all isolates from clinical specimens will hybridize to the probe. For example, there is one strain of CMV, strain Colburn, which was originally isolated from a patient, but which has been shown to be more closely related to simian strains of CMV than HCMV (61,67). It is also known that simian strains can be propagated in human cell culture (61,132), although there are no reports describing human infections with these viruses. The B fragment probe from CMV AD169 needs to be tested against strain Colburn and other simian strains in the event that in the future such strains of CMV are found to produce human disease.

The clinical usefulness of the probe has not yet been demonstrated. Titering of specimens needs to be performed at the time of initial inoculation to determine the number of pfu originally present. The sensitivity of the probe for direct detection of CMV in patient specimens can then be correlated quantitatively with the culture results.

An additional clinical application of the probe would be the identification of CMV in laboratory tissue culture where the titer of the original specimen was too low to detect directly. Presently identification of CMV is based on the rate of appearance of typical CPE and the types of cells in which the virus will grow. Immunological methods of identification are often inconclusive (124). In situ hybridization with either the radiolabelled or biotinylated probe could be performed at specific times after inoculation of replicate tissue culture monolayers. If this technique were sufficiently sensitive, a positive reaction could identify infection with CMV before the appearance of CPE. Positive results would, therefore, be available earlier, and these cultures would require no further laboratory work.

The most clinically significant aspect of nucleic acid hybridization techniques for detection of viruses is the potential for obtaining rapid laboratory results. The method used for the development of the probe during this project allows detection and identification of CMV in 48 h (7). Chou and Merigan (13) referred to the same hybridization protocol but claimed to have results in 24 h. This means the period of hybridization would have to be reduced to less than 24 h. Other reports (81,118), however, support the claim that reliable results can be obtained in 24 h.

Biotin-labelled probes can shorten the time even more, because a high concentration of probe can be used for hybridization without raising the level of non-specific binding (78). Richman et al. (105) reported that their procedure for HSV could be performed in 5 h. Future experiments to demonstrate the clinical application of the CMV probe

developed for this project should address the question of the rapidity with which the probe can detect and identify the virus. Forty-eight hours for detection of CMV as performed during this project is in most cases faster than tissue culture, and the specificity of the probe provides more certain identification of CMV than the appearance of the CPE. The prospect of shortening the time required for complete identification of this virus adds to the potential clinical usefulness of the probe.

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APPROVAL SHEET

The thesis submitted by Nell S. Lurain has been read and approved by the following committee:

Dr. Kenneth D. Thompson, Director
Associate Professor, Pathology/Microbiology, Loyola

Dr. Edward W. Bermes
Professor, Pathology/Biochemistry and Biophysics, Loyola

Dr. Stephen K. Farrand,
Associate Professor, Microbiology, Loyola

Dr. Robert V. Miller,
Associate Professor, Biochemistry and Biophysics, Loyola

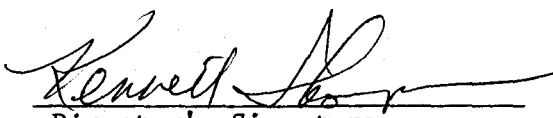
Dr. J. Paul O'Keefe,
Associate Professor, Medicine/Microbiology, Loyola

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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Date



Director's Signature